WEST Search History

DATE: Tuesday, April 01, 2003

Set Name side by side	Query	Hit Count	Set Name result set
DB=USPT,PGPB,JPAB,EPAB,DWPI; PLUR=YES; OP=OR			
L12	L8 and IGG1	1	L12
L11	L8 and parasite?	2	L11
L10	L8 and vesey	0	L10
L9	L8 and oocyst?	0	L9
L8	SDS same boil	35	L8
L7	L5 and cryptosporidium	2	L7
L6	L5 and oocysts	0	L6
L5	SDS and boil	715	L5
L4	L2 and boil	0	L4
L3	L2 and boil?	0	L3
L2	L1 and SDS	179	L2
L1	oocysts	865	L1

END OF SEARCH HISTORY

End of Result Set

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L11: Entry 2 of 2 File: USPT Aug 30, 1988

DOCUMENT-IDENTIFIER: US 4767622 A
TITLE: Method and materials for development of immunological responses protective against malarial infection

Abstract Text (1):
Disclosed are vaccine compositions for use in developing protective immunity against infection by Plasmodium parasites. Soluble proteinaceous immunogens are isolated from the fluid culture medium of in vitro propagated plasmodial species parasites (e.g., P.falciparum) in mammalian erythrocyte culture supernatant or from washes, including hypotonic washes, of cultured erythrocytes parasitized by plasmodium. Immunogens so obtained have molecular weights in the range from about 35,000 daltons to about 85,000 daltons. Two principal immunogens of the invention have molecular weights of about 42,000 and 54,000 daltons, respectively. The water soluble immunogens are administered in a suitable carrier such as isotonic salt solution and in combination with a suitable adjuvant such as saponin or, preferably, aluminum hydroxide. Upon administration to vertebrate animals susceptible to plasmodial infection, vaccines according to the invention provoke immune responses protective against morbidity and mortality caused by, e.g., P.falciparum infection. Disclosed also are procedures for optimization of large scale plasmodial parasite growth in human erythrocyte cultures with accompanying development of late erythrocytic stage parasites in large numbers and optimization of isolatable quantities of culture medium supernatant and wash-derived immunogens.

Brief Summary Text (3):
Human malaria is caused by species of parasitic organisms of the genus Plasmodium. It is transmitted by mosquitoes which ingest sexual forms of the parasite in blood meals. Sporozoite forms of the parasite develop in the mosquito and are transmitted to new host individuals bitten by the insect. In the new host, the sporozoite parasites develop and multiply in an "exoerythrocytic" cycle in the liver without inducing clinical symptoms. The merozoite forms resulting from exoerythrocytic propagation then invade host erythrocytes, initiating an "erythrocytic" cycle of development and prompting the clinical symptoms of malaria. Destruction of red blood cells occurs on a 48-hour cycle with P.vivax, P.ovale and P.falciparum, and every 72 hours with P.malariae. Characteristic chills-fever-sweat malarial symptoms follow this cyclic pattern, being induced by rupture of infected red blood cells by the mature asexual forms (schizonts), releasing merozoites that quickly invade new red cells. In contrast to the exoerythrocytic stage, erythrocytic merozoites induce an array of humoral responses in the host, as demonstrated by appearance of blood serum antibodies detectable by complement fixation, precipitation, agglutination, and fluorescent antibody tests.

Brief Summary Text (8):
A second general approach to immunization has involved use of killed or inactivated merozoite vaccines. See, generally, Cohen, Proc.Royal.Soc.London, 203, pp. 323-345 (1979). Research efforts in this area have been aided greatly by the procedures developed by Trager and Jensen [Science, 193, pp. 673-675 (1976)] relating to continuous culture methods for in vitro propagation of erythrocytic stages of parasites.

Brief Summary Text (10):
Freund's Complete Adjuvant (FCA) or synthetic adjuvants are required for merozoite antigen use and thus constitutes a major deterrent to development of a human vaccine. More recent studies using karyotype-selected Aotus monkeys infected with human P.falciparum, reported prolongation of life in owl monkeys vaccinated with parasite material cultivated in vitro when the synthetic adjuvant muramyl dipeptide was used

instead of FCA. In the rhesus monkey immunization studies, helper T cells, other cell-mediated effector mechanisms, and humoral antibody all appear to be involved. Extracellular merozoites are specifically inhibited by IgG and IgM in the absence of complement. Immunization in Rhesus monkeys reportedly induces complete elimination of parasites after 1-3 weeks, whereas natural immunity following repeated infection and drug cure is associated with chronic relapsing parasitemia. Immunization probably is associated with far fewer soluble circulating antigens than natural infection, which preferentially stimulates suppressor cells or lymphocyte mitogens, all of which favor parasite survival. Among the difficulties associated with immunization with merozoites are risks of contamination of the merozoite vaccine with blood group substances acquired during its cultivation (inducing anemia) and substantial potential problems of vaccine delivery, cost, and acceptance.

Brief Summary Text (13):
McGregor, et al., [Lancet, 1, pp. 881-884 (1968)]; Wilson, et al., [Lancet, 2, pp. 201-205 (1969)]; McGregor, et al., [Trans.R.Soc.Trop.Med.Hyg., 65, pp. 136-151 (1971)]; and Williams, et al., [Af.J.Med.Sci., 4, pp. 295-307 (1972)], relate to demonstrations of the presence of soluble antigens in the plasma of human beings infected with an African strain of P.falciparum. Characterization of the majority of the soluble antigens found in the serum showed them to be heat stable at 100.degree. C. [Wilson, et al., Immunology, 3, pp 385-398 (1973)]. Consequently, they were called "S" antigens. Molecular weights reported for S antigens ranged from 60,000 to 210,000 daltons. Groups of soluble plasmodial antigens not usually found in the serum ("La", "Lb", and "R" antigens), had properties different from S antigens. L antigens were reportedly more immunogenic than S antigens and rapidly reacted with antibody leading to soluble antigen-antibody complexes in the serum [Wilson, et al., Lancet, 2, pp. 201-205 (1969); Houba, et al., Af.J.Med.Sci., 4, pp. 309-317 (1972); and Wilson, et al., Immunology, 3, pp. 385-398 (1973)]. Saul, et al., [Tropenmed. Parasitol., 28, 302-318 (1977)] demonstrated that a soluble protein-containing immunogen could be obtained by washing sonically freed P.berghei parasites with cold saline. Further work by Kreier's group [Grothaus, et al., Infect. and Immunol., 1, pp. 245-253 (1980)], is reported to show that the soluble material was more immunogenic than the intact parasites.

Brief Summary Text (15):
The most recently reported developments in the proposed use of antigenic fragments associated with erythrocytic stages of malarial parasite growth have had their origins at the Wellcome Foundation in the United Kingdom. More specifically, U.K. published Patent Application Ser. Nos. 2,096,893 and 2,099,300 both report that, prior to the development described, "Attempts have been made to define the diversity of protein antigens associated with merozoites. However, no specific antigens capable of inducing a protective response by the host or specifically recognized by such a protective response have been isolated and characterized." Both published applications are said to relate to "protection inducing antigens of parasites of the genus Plasmodium" and both describe the use of affinity separations (involving monoclonal antibodies) to isolate merozoite and schizont form antigens.

Brief Summary Text (16):
As specific examples of practice of the development, both published British applications describe isolation of antigens associated with murine-specific malarial species, Plasmodium yoelii. Briefly summarized, erythrocytes from infected cells of mice are lysed, centrifuged and solubilized with a variety of detergents to yield a supernatant containing erythrocyte soluble proteins, some erythrocyte membrane proteins and an estimated "70% of the parasite antigens". The solubilized material is then passed through an immunoabsorbant column to which specific monoclonal antibodies were bound. The eluate of the antigen/antibody absorption is concentrated and dialyzed to yield non-glycosylated antigens having a molecular weight of 2.35.times.10.sup.5 or 1.95.times.10.sup.5 (assertedly corresponding to merozoite- and schizont-associated antigens). The antigenic isolates are reported to have been successfully used with Freund's Complete Adjuvant to protect mice against lethal challenge P.yoelii parasites. The applications go on to discuss similar attempts to isolate one or more antigens or antigenic fragments from Plasmodium falciparum parasitized erythrocytes, using a correspondingly specific monoclonal antibody. The resulting antigens were tested in vitro for cross-reactivity with P.yoelii antigen but not employed in any in vivo (antibody generation or infectious challenge) work.

Brief Summary Text (17):
Assuming that the projected isolations of P.falciparum schizont and merozoite antigens according to the procedures of U.K. published Patent Application Nos. 2,096,893 and 2,099,300 are as fruitful as the work reported for P.yoelli antigens, it is possible that the solubilized protein isolates may provide useful components for a human vaccine composition. Large scale production of antigenic materials, however, is likely to involve numerous difficulties, including problems in securing large quantities of human blood cells infected with late stages of parasites in large scale solubilization processing of erythrocytes free of red blood cell components, and in large scale maintenance and operation of antibody columns for affinity purification.

Brief Summary Text (18):
As previously noted, development of methods for continuous in vitro propagation of malarial parasites by Trager and Jensen, supra, has markedly assisted in the development of merozoite vaccines and the general study of erythrocytic malarial parasite stages. In a sense, it has also provided a means for detection and isolation of soluble antigens unaffected by the host's metabolic and immune systems. As an example of this type of research, most investigators found maximal quantities of protein material to accumulate in the culture medium during late schizogony and merozoite reinvasion. The possibility of the presence of Plasmodium-associated material in culture supernatant had been reported in cultures of P.knowlesi (Cohen, et al., 1969), P.falciparum (Wilson, 1974; Wilson and Bartholomew, 1975), and P.berghei, (Weissberger, et al., 1979). Wilson and Bartholomew (1975) detected antigens that were heat stable, partially heat labile and heat resistant, termed S, L and R antigens, respectively. Jepson, et al., Acta.Path.Microbiol.Scand., Sect. C, 89, 99103 (1981) reported the isolation of two distinct antigens of the S and R classes from the culture medium of growth of P.falciparum in human erythrocytes. The isolation procedure involved immunoabsorbant techniques and is said to have yielded approximately 3 milligrams of the two antigens from 800 milliliters of culture medium. The results were said to "show promise for further attempts to isolate other antigens from the culture medium, and for obtaining knowledge about the chemistry and biology of the isolated antigens". Similarly, Thelu, et al., WHO Bulletin, 60, pp. 761-766 (1982) reports on the chromatographic isolation of an "Antigen E" from cultured P.falciparum and correlations between this substance and antigens in sera of human patients in endemic areas.

Brief Summary Text (20):
It has consistently been the case of the nonsporozoite materials displaying potential as anti-malaria vaccine components are so weakly immunogenic as to absolutely require the use of oil and water adjuvants such as Freund's Complete Adjuvant (FCA) to develop any effect. Such adjuvants are not accepted for use in humans. In anticipation of the discovery of truly protective anti-malarial antigens, substantial and relatively continuous efforts have been made in the screening of existing adjuvants and the development of new adjuvants for vaccine use. U.S. Pat. No. 3.849,551, for example, proposes the use of Mycobacteria bovis, strain Calmette-Guerin bacillus (BCG) as an adjuvant for malaria vaccines, and Schenkel, et al. [J.Parasitol., 61, pp. 549-550 (1975)] propose mixtures of BCG with Adjuvant 65 as providing even more beneficial results. Desowitz [Experimental Parasitology, 38, pp. 6-13 (1975)] provided a comprehensive screening study of various adjuvants used with a P.berghei, blood-derived soluble antigens. Among the many results of tne study was the conclusion that ferric alum and aluminum chloride precipitated antigens were non-immunogenic while aluminum-alum-precipitated antigens might be protective. As previously noted, Mitchell, et al., supra, studied adjuvant effects for merozoite antigens and concluded that muramyldipeptide in mineral oil was partially effective in some studies and saponin was demonstrably effective in others. Siddiqui, et al. [Nature, 289, pp. 64-66 (1981)] reports on "effective immunization of monkeys with killed parasites and N,N-dioctadecyl-N',N'-bis(2-hydroxyethyl-propanediamine) McColm, et al., supra, reported the testing of numerous adjuvants with a killed parasite vaccine and concluded that none were as effective as saponin, although FCA, aluminum hydroxide and C.parvum augmented immunity considerably. Correspondingly, the aforementioned U.K. published Patent Application Nos. 2,096,893 and 2,099,300 report use of FCA in vaccination tests designed to illustrate potential utility for the isolated antigen, but note that "convenient" adjuvants for use in vaccines include saponin, C.parvum and aluminum hydroxide.

Brief Summary Text (23):
The present invention provides, for the first time, anti-malarial vaccine compositions of demonstrable in vivo efficacy which are formulated through use of one or more proteinaceous immunogens readily isolatable from (1) the host erythrocyte-free and parasite-free supernatant fluid medium of in vitro cultured growth and proliferation of Plasmodium parasites in susceptible erythrocytes, and/or (2) washes, including hypotonic washes, of erythrocytes from parasite-infected cultures.

Brief Summary Text (26):
In another of its aspects, the present invention provides a series of procedures for the optimization of the in vitro growth of P.falciparum parasites in long-term human erythrocyte cultures leading to the large scale production of human erythrocytes infected with late stage parasites and optimal production of culture medium supernatant and wash-derived immunogens. Briefly, cultures are initiated and sub-cultures are developed through use of cells and serum pre-screened for matching histocompatibility factors (e.g., major blood groups) and incompatibility of serum to erythrocytes in the context of "warm" and "cold" antibody/antigen reactions.

Brief Summary Text (29):
The following illustrative examples of practice of the present invention are directed to presently preferred procedures for obtaining the active proteinaceous immunogens employed according to the invention, for formulating vaccine compositions through use of the antigens with selected adjuvants and for using vaccines so formulated to develop protective immunity in susceptible vertebrates against infection by plasmodial parasites.

Brief Summary Text (30):
More specifically, Examples I and II are directed to procedures for in vitro cultivation of P.falciparum parasites in human and non-human erythrocyte cultures. Example III is directed to procedures for isolating antigens from tne fluid culture medium of parasite propagation and from washes of infected erythrocytes. Example IV is directed to characterization of physical and in vitro immunological properties of antigens so obtained. Example V describes the preparation of specific vaccine compositions according to the invention which employ the culture-derived antigen along with exemplary saponin and aluminum hydroxide adjuvants.

Detailed Description Text (8):
Another initial series of procedures involved in in vitro cultivation of P.falciparum in mammalian erythrocytes relates to pre-screening cells and serum to be employed in the cultures so that in vitro propagation of parasites may be optimized. A first measured parameter of candidate erythrocytes is their osmotic stability. Briefly, approximately 5 ml of each potential uninfected cell source is washed three times in serum-free culture medium. If any cell lysis occurs during any wash, the cells are ciscarded as too fragile for culture purposes.

Detailed Description Text (9):
All candidate sera and plasma are screened for major blood group histocompatibility with both infected and uninfected cells to be employed in cultures by simple admixture in microtiter plates at room temperature and microscopic monitoring for agglutination. Typically, adverse effects use of serum sources containing, e.g., anti-A antibodies with A cells will be avoided by these procedures. Room temperature screening for agglutination also serves to eliminate serum sources possessing so-called "cold antibodies" (to M and P erythrocyte antigens). If no clotting or agglutination occurs, plates are covered, placed in a humidified incubator at 37.degree. C. and monitored for agglutination at 1 hour, 3 hours, and overnight to detect agglutination which may be due to "warm antibodies", e.g., anti-Fy.sup.a -Fy.sup.b, anti-JK.sup.a, and anti-K. It has been determined that screening for compatability in terms of Rh factors is not absolutely required for success of in vitro cultivation proceduces according to the invention. Parasites appear to propagate well, for example, in A+ or A- cells, but show a preference for A.sup.- cells.

Detailed Description Text (13):
The method of parasite cultivation employed is a modification of the described by Trager and Jensen, supra, Parasites are cultured in 35.times.10 mm Falcon tissue

culture dishes (1.5 ml of the 10% suspension/dish) anc incubated at 37.degree. C. in candle jars or in Corning 25 cm.sup.2 tissue culture flasks (5.0 ml/flask) under a precision gas mixture atmosphere of 6% O.sub.2, 10% CO.sub.2, and 84% N.sub.2. The culture supernatant medium is replaced once or twice daily; medium containing 10% monkey serum is used to maintain cultures.

Detailed Description Text (15):
Gentamicin (Gentocin, Schering Corp., Kenilworth, N.J.) in a concentration of 0.1 .mu.l/ml to 1.0 .mu.g/ml of complete medium was employed as an anti-microbial agent. Concentrations greater thjn 10 .mu.g per ml were toxic to parasites.

Detailed Description Text (18):
Several culture lines were initiated and terminated after varying growth periods ranging from 5 to 21 days. Daily microscop9c examination of P.falciparum cultures revealed multiplication of the organism. The beginning parasitemia of 0 5%-increased to an average of 4% within 72 hours of incubation. Subcultures were made when parasitemias were between 2% and 4% in order to provide for continuation of the line. On occasion, parasites were grown 5 or 6 days without subculture resulting in an average peak parasitemia of about 8.5%. An active multiplcation of the Organism was also detected by the appearance of all growth forms of Plasmodium, beginning with ring forms, through trophozoite, schizont and merozoite stages. While Minimum Essential Medium (Eagle 138 Special, Gibco Laboratories, Grand Island, N.Y.) also supported the growth of P.falciparum in squirrel monkey erythrocytes, there was no apparent advantage of using this medium over RPMI 1640. Medium 199 supplemented with penicillin-streptomycin did not support the growth of P.falciparum Indochina I in the primate culture system.

Detailed Description Text (21):
For antigen production in a human system, several large vials of stabilate of the desired strain(s) of P.falciparum are reactivated inro 5 ml or 15 ml flasks using a precision gas mixture. Simultaneously, each strain of P.falciparum is cultured in duplicate 35 mm dishes using candle jar methods as a back-up system. Similarly, two dishes conraining uninfected erythrocytes are cultured. Within three days to one week subsequent to reactivation, infected erythrocytes are subcultured into six to ten flasks (150 cm.sup.2) in addition to a continuous flow vessel using precision gas. Plasmodial strains are subcultured alternately every third followed by every fourth day (e.g., two times each week). Within three subcultures, 80 ml of parasitized erythrocyte suspension optimally generates 800 ml followed by 8,000 ml of culture supernatant using the flow vessel alone. Using the latter vessel, the work is accomplished with a minimum of labor. To allow for maximum peak parasitemia without loss of culture lines, parasites are subcultured to lower parasitemia levels of about 0.2% ar the beginning of the fourth day growth period, and alternately are subcultured down to about 0.4% prior to the third day growth period. Consequently, approximately the same peak parasitemia level is being maintained throughout the cultivation period.

Detailed Description Text (22):
The following specific cultural procedures and gas mixtures have been used in order to accomplish antigen production in human cell systems. Parasites grown in microtiter plates or in 35 mm dishes are placed in a candle jar in order to obtain the reduced oxygen tension necessary for the growth of Plasmodium. Parasites grown in 25 cm.sup.2 flasks, 75 cm.sup.2 flasks, 150 cm.sup.2 flasks or flow vessels, are grown under a precision gas mixture of 6% oxygen, 10% CO.sub.2, and 84% nitrogen. In the flow vessel, the incubator is not flooded with the precision gas mixture; the gas line is guided directly into the flow vessel. In the plates or in the flasks, culture suspensions are maintained in a stationary position. In contrast, in the flow vessel, following the mixing of infected and uninfected erythrocytes with media on subculture days, the flow vessel is rocked at a very slow speed for approximately twenty minutes prior to stopping it and bringing it into a stationary position. For optimal parasite growth the flow vessel should be rocked only twice daily for fifteen minutes each time, and not continuously, to allow for the proper mixing of nutrients.

Detailed Description Text (42):
Culture supernatants were concentrated by pressure dialysis using the Amicon diaflo system. Concentrated culture supernatants were lyophilized, reconstituted in distilled

water,·concentrated 2.times. and dialyzed with phosphate buffer containing SDS. One hundred ml of the 2.times. concentrated supernatant was incubated for three hours at 37.degree. C. Samples were brought to room temperature. Five ml of 2-mercaptoethanol and 5 ml of 0.25% bromophenol blue were added to the 100 ml of the protein mixture. Seven ml was loaded onto the track and pre-electrophoresed for one-half hour at 150 milliamps. Following preelectrophoresis, samples were electrophoresed at 190 miliamps for 41/2 hours prior to staining. In optimization of the system, it was found that in order to pick up the antigens, it is better to run the samples for three hours at 37.degree. C. than to boil for three minutes at 100.degree. C. Also, in examining concentrations of supernatants ranging from unconcentrated supernatant to supernatant that was concentrated 34 times, it was found that the optimal concentration of the supernatant for detection of these antigens is 2.times..

Detailed Description Text (66):
Animals were rested and acclimated for a minimum of three weeks prior to experimental use. During this time, animals were processed through all examinations as required by the public health authorities. These included testing for exposure to Mycobacterium. One tenth ml of tuberculin (Jensen Salsbury Laboratories, Kansas City, Mo.), was injected intradermally above the upper eyelid. The test was read at 24, 48, and 72 hours for the presence of positive delayed type hypersensitivity reaction. Animals were screened for the presence of blood parasites by microscopic examination of thick and thin Giemsa-stained blood films. The presence of intestinal parasites was ascertained by examination of fecal samples.

Detailed Description Text (71):
The number of parasites/mm.sup.3 of blood in challenged animals was determined by enumerating free P.falciparum parasites on Giemsa-stained thick smears. A 5 .mu.l sample of infected blood was obtained from the calf portion of the leg of infected animals by cleaning the shaved area with 95% ethanol, sticking with a sterile lancet and drawing blood to the 5 .mu.l mark in a micro-sampling pipet (Corning, Arthur H. Thomas Co., Philadelphia, Pa.). Blood was quickly transferred to a glass slide and spread uniformly in an area of specific length and width. Unfixed, Giemsa-stained smears were examined and parasites enumerated with the aid of a Howard grid. Five sweeps were made across the width of each smear and the contents of the entire grid were counted. The number of parasites counted was converted into number of parasites/mm.sup.3 of blood.

Detailed Description Text (86):
Several days prior to vaccination and during post-vaccination and post-challenge periods, the rectal temperature of the animals and their clinical health were determined daily, while hematocrit levels and IFA determinations were made at intervals of approximately one week. Enumeration of the parasite in the peripheral blood samples was made microscopically using Giemsa-stained thick smears. In addition to the clinical protection, the evidence of protective immunity in vaccinated animals was revealed by active reticulocytosis using blood smears stained with new methylene blue, stable hematocrit levels, the pattern of IFA response following challenge, and the presence of structurally abnormal parasites situated intra- and extra-erythrocytically.

Detailed Description Text (92):
Parasitemias calculated on the basis of thick blood smear examination are expressed as the total parasite number/mm.sup.3 of blood and are shown in FIG. 3. In the scale shown in FIG. 4 maximum parasite number (45,155 parasitized mm.sup.3 blood) is equal to 100%. The negative control animal demonstrated two parasitemia peaks with 45,155 and 41,000 parasites/mm.sup.3 of blood observed on days 17 and 24 post-challenge, respectively. During the majority of the post-challenge period prior to recovery, the parasitemia levels in vaccinates were only about 25% of that seen in the negative control with the exception of one reading period where parasitemia was about 50% of that seen in the negative control. Only negligible parasite numbers were detected in the positive control monkey.

Detailed Description Text (93):
Aside from the difference in parasitemias between vaccinates and the susceptible control, vaccinates exhibited a delayed prepatent period with parasites detectable on thin smears long after acute disease symptoms were observed in the control monkey.

Detailed Description Text (94):
Microscopic examination of the parasites in vaccinated animals using Giemsa-stained thin blood films showed that more than 50% of the parasites were structurally abnormal and that many of these were situated extracellularly. The presence of such abnormal parasite forms and the evidence of reticulocytosis in the vaccinates and the preimmunized control, along with other pertinent parameters during the post-challenge period are presented in FIG. 4. The presence of abnormal parasites was limited to the vaccinated animals only. Compare, for example, the Giesma-stained thin blood films of immunized monkeys in FIG. 9, plates A, B and C with that of blood from a non-vaccinated, splenectomized monkey shown in FIG. 9, plate D.

Detailed Description Text (116):
FIG. 8 sets out information concerning extent of post-challenge parasitemia in the eight experimental animals. Data concerning peak parasitemia correlated to time elapsed after challenge as determined by thin and thick blood smear procedures is set out in Table 12, below. While single parasites were occasionally seen in thin smears of carrier animals (Nos. 1, 2 and 3), parasitemia was considered to be "zero".

Detailed Description Text (122):
The most prominent and rapid inhibition of parasite reinvasion was achieved with the serum of the Example VII monkey immunized with soluble antigens fortified by the aluminum hydroxide adjuvant. The inhibiting anti-parasite antibody effect was well illustrated by the appearance of deformed and extra-cellularly-occuring parasites as revealed by microscopic examination of Giemsa-stained thin blood films. It should be noted that the serum antibody generated in Example VII reacted in double diffusion in gel with soluble antigen from human culture as discussed in Example IV(A).

Detailed Description Text (124):
Another study was conducted to examine growth inhibition effects in a heterologous strain system. In this case, sera produced in response to vaccination with soluble supernatant antigens derived from cultures of P.falciparum, "Geneve" strain, which originated in the Senegal region of Northwest Africa. As indicated in FIG. 6, antiserum of a monkey vaccinated with the Indochina I strain supernatant antigen and aluminum hydroxide adjuvant (Example VII) showed a considerable degree of growth inhibition of the Geneve strain parasites in comparison to normal human and monkey sera, with growth inhibition characteristics being essentially on par with those of serum of a recovered carrier monkey previously infected with the Indochina I strain.

Detailed Description Text (125):
The foregoing Examples are believed to clearly illustrate numerous interrelated aspects of the present invention having to do with the in vitro propagation of plasmodial parasites and development of protective vaccines employing materials generated in the course of such propagation. The disclosures of Example 1 relating to "screening" procedures, when combined with subsequent disclosures of the consistent development of large populations of parasitized erythrocytes, illustrate that aspect of the invention which constitutes a substantial improvement in propagative procedures of the prior art. Whenever initial continuous cultured growth of parasites is effected by addition of infected erythrocytes to susceptible, uninfected erythrocytes in a medium including serum, practice of the invention dictates use of combinations of erythrocytes and serum from blood sources having the same blood group (saline reactive) antigens, and antigens reactive with atypical antibodies. In this manner, common antigen/antibody agglutination reactions are entirely avoided in both the initial cultures and all subsequent subcultures. Not only do these procedures immensely facilitate production and isolation of the soluble antigens employed in vaccines of the invention, they also make available large populations of late erythrocytic-stage parasites. Such parasitic forms, including schizonts and merozoites, may be advantageously employed to provide solubilized antigens such as those described in U.K. published Patent Application Nos. 2,096,893 and 2,099,300. Practice of the improved propagative methods of the present invention is thus seen to facilitate any attempt to isolate those insoluble antigenic materials which may be associated with plasmodial parasites and parasite fractions.

Detailed Description Text (126):
In another of its aspects, the present invention provides the first verified

demonstration ever that in vitro cultivation of plasmodial parasites can consistently generate water-soluble antigenic materials which, upon isolation from culture medium supernatant and wash preparations, can generate in a vaccinated primate a protective immune response to a massive malarial parasite challenge. It is noteworthy that the protective effects of vaccination according to the invention are enduring in nature. Animal No. 4 of Example VIII was unequivocally protected against a lethal parasitic challenge despite the passage of seven months between vaccination and challenge and despite the decrease in level of circulating antimalarial antibodies to less than that commonly found in carrier animals. The development of such a long-term, anemnestic response has no counterpart in the decades of vaccination studies which preceded the present invention.

Detailed Description Text (129):
The procedures of the above illustrative examples involve the use of Plasmodium falciparum parasites. They were designed to establish the closest possible analogy in test procedures to plasmodial infections in, and anti-plasmodial vaccination of, human beings. It will be understood, however, that the various methods and materials of the invention may be easily adapted to use with other human-specific plasmodial species including P.vivax, P.ovale and P.malariae, as well as with those species which are specific for non-human hosts.

Other Reference Publication (10):
Science, vol. 193, Aug. 20, 1976, pp. 673-675, Trager, William et al., "Human Malaria Parasites in Continuous Culture".

CLAIMS:

1. A vaccine composition for use in developing a protective immune response in a vertebrate animal susceptible to infection by Plasmodium flaciparum parasites, said composition comprising:

(1) an immunologically effective amount of one or more water soluble proteinaceous immunogens having respective molecular weights within the range of about 35,000 and about 85,000, as determined by SDS-PAGE, produced in the course of the in vitro cultured growth and proliferation of Plasmodium falciparum parasites in a susceptible host erythrocyte cell culture and isolated from the host and parasite cell and cell fragment-free medium of such growth or host and parasite cell and cell fragment-free washes of infected host cells in such culture; and

(2) an immunologically effective amount of immunologically acceptable carrier and adjuvant materials.

6. A method for protecting a susceptible vertebrate against infection by Plasmodium falciparum parasites comprising administering a vaccine composition comprising:

(1) an immunologically effective amount of one or more water soluble proteinaceous immunogens having respective molecular weights within the range of about 35,000 and about 85,000, as determined by SDS-PAGE, produced in the course of the in vitro cultured growth and proliferation of Plasmodium falciparum parasites in a susceptible host erythrocyte cell culture and isolated from the host and parasite cell and cell fragment-free medium of such growth or host and parasite cell and cell fragment-free washes of infected host cells in such culture; and

(2) an immunologically effective amount of immunologically acceptable carrier and adjuvant materials.

L4 ANSWER 1 OF 14 USPATFULL

ACCESSION NUMBER:	2003:47512 USPATFULL
TITLE:	41 kDa **Cryptosporidium** parvum **oocyst** wall protein
INVENTOR(S):	Jenkins, Mark C., Davidsonville, MD, United States Fayer, Ronald, Columbia, MD, United States Trout, James, Columbia, MD, United States
PATENT ASSIGNEE(S):	The United States of America as represented by the Secretary of Agriculture, Washington, DC, United States (U.S. government)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6521229	B1	20030218
APPLICATION INFO.:	US 2001-888655		20010626 (9)

RELATED APPLN. INFO.:	Division of Ser. No. US 1999-451117, filed on 30 Nov 1999, now patented, Pat. No. US 6277973
DOCUMENT TYPE:	Utility
FILE SEGMENT:	GRANTED
PRIMARY EXAMINER:	Housel, James
ASSISTANT EXAMINER:	Winkler, Ulrike
LEGAL REPRESENTATIVE:	Silverstein, M. Howard, Fado, John D., Rabin, Evelyn M.
NUMBER OF CLAIMS:	6
EXEMPLARY CLAIM:	1
NUMBER OF DRAWINGS:	14 Drawing Figure(s); 12 Drawing Page(s)
LINE COUNT:	1528

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

TI 41 kDa **Cryptosporidium** parvum **oocyst** wall protein

AB . . . for the immunization of animals against cryptosporidiosis. The proteins are effective for the immunization of a variety of animals against **Cryptosporidium** parvum, particularly for the production of hyperimmune colostrum that may be used to confer passive immunity against the parasite. Isolated. . .

SUMM **Cryptosporidium** parvum is a protozoan parasite that has been implicated in numerous outbreaks of diarrheal disease in the human population. This. . . are specific for the native 41 kDa protein which specifically identifies C. parvum and thus distinguishes C. parvum from other **Cryptosporidium** species, to generate hyperimmune serum or colostrum for use in enhancing the anti-cryptosporidial response of young or immunocompromised individuals, and in vaccine development, to protect individuals from **Cryptosporidium** infection.

SUMM **Cryptosporidium** is a protozoan that can cause acute, severe, self-limited disease in immunocompetent individuals and severe chronic diarrhea in immunocompromised individuals.. . . hosts such as persons afflicted with AIDS. Development normally takes place in the intestinal epithelium and the transmissible stage, the **oocyst**, is excreted in the feces. In immunocompromised patients, cryptosporidiosis is not necessarily self-limiting and sites other than the small intestine,. . . liver, pancreas, gall bladder, appendix, colon, rectum, and conjunctiva of the eye, may be affected (Fayer et al. 1997. In **Cryptosporidium** and Cryptosporidiosis, R. Fayer, Ed., CRC Press, New York, N.Y., page 29). Cryptosporidiosis is also a major disease of dairy and beef calves in the United States. Although a number of species of **Cryptosporidium** have been described, only C. parvum causes disease in both humans and calves.

SUMM . . . of the parasite was traced to contaminated drinking water supplied by a municipal water treatment utility. Such widespread occurrence of **Cryptosporidium oocysts** in raw and treated drinking water supplies throughout the USA has raised concern that low-level endemic waterborne **Cryptosporidium** infections may occur commonly.

SUMM **Cryptosporidium** is transmitted through animal contact, person-to-person contact, and contaminated food and water. The C. parvum infection is initiated by the ingestion of **oocysts**, the excystation of **oocysts** with release of sporozoites and the

invasion of gut epithelial cells by sporozoites. Thereafter, the intracellular forms mature and release. . . sexual cycle. The sexual cycle of C. parvum also occurs in the gut and results in the production of sporulated **oocysts**, some of which may excyst before being shed. In persistent infection of an immunocompromised host, both the merozoite and the endogenously produced sporozoite may contribute to the ongoing invasion by C. parvum. **Cryptosporidium** spp. are resistant to standard disinfection processes and remain infectious for long periods of time in the environment at a wide range of temperatures. This resistance is imparted by the hard outer covering of the **oocyst** wall that surrounds the infectious stage of the parasite, i.e., sporozoites.

SUMM The detection of **Cryptosporidium** parvum **oocysts** in environmental samples usually relies on one of three different techniques--vital dye staining (e.g., Modified Ziehl-Neelsen acid fast staining), direct or indirect immunofluorescence staining (IFA), or enzyme immunoassay (EIA) using **Cryptosporidium**-reactive antibodies. Differences in the relative sensitivities of these assays have been noted (Garcia et al. 1997. J. Clin. Micro. 35. . . et al. 1995. J. Clin. Microbiol. 33: 416-418). The majority of immunocompetent patients, when initially symptomatic, have large numbers of **oocysts** present in their stools and their condition can be confirmed with a number of procedures; however, as the acute infection resolves, the patient becomes asymptomatic and the number of **oocysts** dramatically decreases (Garcia et al. 1997, supra). Low numbers of **oocysts** makes identification of C. parvum as the causative agent difficult. The high sensitivity of anti-**Cryptosporidium** monoclonal antibodies (mAbs) most certainly aids detection of **Cryptosporidium** in fecal or environmental samples; however, their use does not ensure the specific detection of C. parvum, the only species that represents potential public health threats. Crypto-sporidium **oocysts** shed by a variety of captive and wild homoiothermal and poikilothermal animals contaminate the surface water and water supply. In the absence of C. parvum-specific mAbs, such **oocysts** can be misinterpreted as C. parvum **oocysts** potentially pathogenic for humans based on their identification as **Cryptosporidium oocysts** by crossreactive antibodies, i.e., antibodies that react with more **Cryptosporidium** species than C. parvum (Graczyk et al. 1996. Am. J. Trop. Med. Hyg. 54(3): 274-279). Similarly, diarrhea in patients may. . . from C. parvum under circumstances where an organism other than C. parvum is the causative agent and the patient carried **Cryptosporidium oocysts** (not C. parvum) from contacts not related to the diarrheal disease, i.e., environmental contacts. This problem is of particular concern. . . of treated water destined for human consumption. None of the available immunoassays can differentiate C. parvum from other species of **Cryptosporidium** that are not infectious for mammals. The inability to sensitively detect and differentiate **Cryptosporidium** at the level of species or subspecies (strain) is a recognized constraint on our understanding of the natural history, epidemiology, and zoonotic potential of **Cryptosporidium** isolates and therefore makes the assessment of the public health risk posed by **oocyst** contamination of water or foods difficult (M. J. Arrowood. 1997. In **Cryptosporidium** and Cryptosporidiosis, R. Fayer, Ed., CRC Press, New York, N.Y., page 56).

SUMM Confirmatory diagnosis of cryptosporidiosis in patients is often carried out by assaying sera for recognition of specific **Cryptosporidium** antigens (Frost et al. 1998. Epidemiol. Infect. 121: 205-211). Several low molecular weight C. parvum **oocyst** antigens, such as 15 kDa, 17 kDa, and 23 kDa proteins, appear to be useful for identifying the presence of **Cryptosporidium**. The immunogenicity of 15, 17, and 23 kDa antigens and somewhat higher M.sub.r antigens (e.g., 32, 47 kDa) has been observed in other mammalian species infected or immunized with C. parvum **oocysts** (Lorenzo et al. 1995. Vet. Parasitol. 60: 17-25; McDonald et al. 1992. Parasite Immunol. 14: 227-232; Nina et

al. 1992.. . . Parisitol. 80:137-147; Reperant et al. 1994. Vet. Parasitol. 55: 1-13). However, laboratory studies have shown these immunodominant antigens and other **oocyst**/sporozoite proteins to be present in other **Cryptosporidium** species (Nina et al. 1992, supra; Tilley et al. 1990. Infect Immun. 58: 2966-2971); therefore, their presence is not indicative. . . parvum infection. This cross-reactivity of immunodominant antigens may explain why commercial antibody-based tests cannot differentiate C. parvum from species of **Cryptosporidium** that are not infectious for humans.

SUMM . . . antiprotozoal drugs licensed for animal use have been approved for prophylaxis or therapy of cryptosporidiosis (Fayer et al. 1997. In **Cryptosporidium** and Cryptosporidiosis, R. Fayer, Ed., CRC Press, New York, N.Y., pages 20 and 30-31). Several researchers have shown, however, that in calves, mice and humans, administration of hyperimmune bovine colostrum, prepared by immunizing cows with extracts of C. parvum **oocysts**, can effectively confer passive immunity against cryptosporidiosis (Fayer et al. 1989. J. Parasitol. 75(1):151-153; Fayer et al. 1989. J. Parasitol.. . . colostrum have been reported to ameliorate C. parvum infection in AIDS or other immunocompromised patients. Hyperimmune bovine colostrum prepared against **oocysts** contains neutralizing antibodies that recognize epitopes expressed by all life-cycle stages of **Cryptosporidium**.

SUMM . . . and either prevent or lessen the severity of infection in animals. The characteristics of many mAbs which specifically react with **Cryptosporidium** have recently been reviewed; many are neutralizing (Riggs. 1997. In **Cryptosporidium** and Cryptosporidiosis, R. Fayer, Ed., CRC Press, New York, N.Y., Chapter 6). In some instances, the epitope recognized by the. . .

SUMM . . . recombinant antigens can be used to prepare monoclonal antibodies (mAb) which selectively identify or which are specific for C. parvum **oocysts**. These mAbs can be used in ELISA and in IFA to detect the parasite in diarrhea or in water samples similar to **Cryptosporidium** spp.-binding mAb used in commercial diagnostic kits (e.g., Merifluor). Third, the recombinant antigens can be used to generate hyperimmune serum. . . particularly RT-PCR. PCR assays have been developed in a number of laboratories, including our own, to detect less than 10 **oocysts** in a spiked water sample, but the previously identified primers, as well as primers generated from the nucleotide sequences of SEQ ID NO:1, amplify DNA from all species of **Cryptosporidium**. However, primers can be used in RT-PCR to specifically identify transcription of C. parvum-specific proteins.

SUMM We have now discovered a novel recombinant DNA clone designated rCP41 which encodes an **oocyst** wall protein of **Cryptosporidium** parvum, which may have immunodiagnostic potential for cryptosporidiosis as well as potential for use in the production of hyperimmune colostrum. . .

SUMM . . . an immune response specific for C. parvum. This invention, in addition to the above, also encompasses a method of diagnosing **Cryptosporidium** infection of a subject, comprising: contacting a body fluid obtained from the subject with the peptide of this invention; and detecting any selective binding of the peptide to any anti-**Cryptosporidium** antibodies in the body fluid.

SUMM In particular, this invention comprises a method of diagnosing **Cryptosporidium** infection of a subject, comprising: contacting a body substance obtained from the subject with an anti-C. parvum antibody; and detecting. . .

SUMM Further, as a public health issue, there is a need for a method to identify and enumerate the presence of **Cryptosporidium** parvum in water. This invention comprises a method of identifying the presence of C. parvum in water, comprising: contacting a. . .

SUMM Another object of the invention relates to a method of inhibiting or ameliorating a **Cryptosporidium** infection in an individual comprising administering to an individual in need of such treatment an amount of an anti-CP41 or. . .

SUMM . . . sequence shown in SEQ ID NO:2 and wherein said protein is antigenic and effective to elicit an immune response against

Cryptosporidium parvum in a host animal and a second unrelated peptide expressed by a regulatory DNA segment operably coupled to the. . . sequence shown in SEQ ID NO:2 and wherein said protein is antigenic and effective to elicit an immune response against **Cryptosporidium** parvum in a host animal operably coupled to yet another unrelated polypeptide sequence (different from the regulatory protein). It is. . .

SUMM Also part of this invention is a **Cryptosporidium** diagnostic kit, comprising anti-**Cryptosporidium**-specific antibodies; and instructions for the use of the kit.

SUMM Furthermore, this invention also provides a **Cryptosporidium** diagnostic kit, comprising the proteins and peptides of this invention; and instructions for use of the kit.

DRWD FIG. 1 shows the immunostaining of **SDS**-PAGE fractionated native **Cryptosporidium** parvum (Cp) or C. baileyi (Cb) **oocyst** protein, or NiNTA-purified (P) and unpurified (IP) recombinant CP41 protein (rCp41) with rabbit sera to whole C. parvum **oocyst** protein (R.alpha.CpOO), native (R.alpha.NATIVE Cp41) or recombinant (R.alpha.RECOMBINANT Cp41) Cp41antigen, or normal control rabbit sera (NRS). MrS, molecular weight standards.

DRWD FIG. 2 shows the DNA sequence and the predicted amino acid sequence of CP41 DNA clone isolated from **Cryptosporidium** parvum **oocysts**. Putative ATG start sites are indicated in bold.

DRWD FIG. 3 shows PCR amplification of the CP41 sequence from genomic DNA (equivalent to 10.sup.3 **oocysts**) from a bovine isolate of **Cryptosporidium** parvum (Cp-bov), a human isolate of C. parvum (Cp-hu), C. baileyi (Cb), or C. wrairi (Cw). Kbp, .phi..times.174 DNA standards.

DRWD FIG. 4 shows the molecular analysis of **Cryptosporidium** parvum for presence of CP41 sequence in genomic DNA using PCR, and for CP41 mRNA in total RNA using RT-PCR. The PCR assay was performed on DNA equivalent to 10.sup.3 C. parvum **oocysts** (CP DNA). The RT-PCR was performed on total RNA equivalent to 5.times.10.sup.5 C. parvum **oocysts** stored at 4.degree. C. for 6 mo. (CP1), 3 mo. (CP2), or 1 mo. (CP3). +, Superscript reverse transcriptase (Rtase). . .

DRWD FIG. 5 shows the immunofluorescence staining of **Cryptosporidium** parvum **oocysts** with rabbit antisera prepared against native CP41 protein (A), or recombinant CP41 antigen (B), or with normal control rabbit sera. . .

DRWD FIG. 6 shows immunoelectron microscopy staining of **Cryptosporidium** parvum **oocysts** with rabbit antisera prepared against native CP41 protein (A), or recombinant CP41 antigen (B). Bar=100 nm.

DRWD FIG. 7 shows serological titers against recombinant CP41 (.box-solid.--.box-solid.) and native **Cryptosporidium** parvum **oocyst** protein (.tangle-solidup.--.tangle-solidup.) in four calves (A-D) exposed to a natural C. parvum infection and one calf (E) exposed to an experimental natural C. parvum infection as revealed by ELISA. Arrow indicates first day of C. parvum **oocyst** shedding.

DETD . . . 41 kDa protein, CP41, and rCP41, the recombinant 36 kDa and 28 kDa proteins, all of which are specific for **Cryptosporidium** parvum and the nucleic acid sequences that encode these proteins. Antibodies resulting from immunizations with the recombinant 36 and 28. . .

DETD . . . species-specific PCR test based on genomic DNA, but that the CP41 primers are useful for species-specific RT-PCR analysis of total **Cryptosporidium** RNA (Example 14).

DETD . . . proteins of this invention in monomeric or multimeric form can be incorporated into vaccines capable of inducing protective immunity against **oocysts**/sporozoites of C. parvum. The peptides or proteins of this invention can be administered as multivalent subunit vaccines in combination with other antigens of C. parvum. For example, they may be administered in conjunction with other **oocyst** /sporozoite components of C. parvum. Furthermore, it will be understood that peptides specific for a plurality of C. parvum stages and **Cryptosporidium** species may be incorporated in the same vaccine

composition to provide a multivalent vaccine. In addition, the vaccine composition may. . .

DETD . . . al., referred to above. In addition, the method used by Jenkins et al. to successfully obtain high levels of colostral anti-**Cryptosporidium** antibodies is described in U.S. Pat. No. 5,591,434, the contents of which are herein incorporated by reference. Briefly, purified recombinant. . .

DETD Also an important part of this invention is a method of diagnosing **Cryptosporidium** infection, that comprises contacting a body substance with an anti-**Cryptosporidium** antibody having specificity for the polypeptide of this invention; and detecting any selective binding of the antibody to any antigenic **Cryptosporidium** peptides present in the body substance. The anti-**Cryptosporidium** antibodies may be monoclonal or polyclonal. Also provided herein is a method of detecting the presence of **Cryptosporidium** parvum in water samples. The detection of the antibody-polypeptide complex may be conducted by any method known in the art.. . .

DETD . . . and fluorescence signal on a flow cytometer. Positive control seeded samples have shown a linear correlation with the number of **oocysts** recovered from the gradients (Arrowood et al. 1995, supra). Flow cytometric analysis of stool samples from infected bovine and human. . .

DETD . . . or concentrate the low numbers of C. parvum in the sample. For example, C. parvum can be concentrated using biotin-labeled anti-**Cryptosporidium**-specific mAbs, together with anti-biotin-labeled magnetic beads. Because of the microscopic size of the MAC beads, any **oocysts** positively selected by this method can then be stained with FITC-anti-rCP41 mAb, specific for C. parvum, and rapidly and specifically. . .

DETD In a most preferred embodiment of the presently claimed diagnostic methods for identifying presence of C. parvum **oocysts**, the method comprises the steps of: collecting a water sample; isolating an **oocyst**-rich fraction from the water sample by concentration with immunomagnetic methodology; staining the concentrated **oocysts** with C. parvum-specific mAb, and determining the actual number, if any, of C. parvum **oocysts**.

DETD Additionally provided herein is a method of diagnosing **Cryptosporidium** infection, that comprises contacting a body substance with one of the polypeptides of this invention; and detecting any selective binding of the polypeptide to any anti-**Cryptosporidium** antibodies in the body substance. As in the previous case, the present antibody-polypeptide binding complex may be detected by a. . .

DETD Still part of this invention is a kit for the diagnosis of **Cryptosporidium** infection, that comprises the peptide(s) of this invention; and instructions for use of the kit. This kit may be utilized. . . with cryptosporidiosis. Even at the early stages where the parasite is commencing invasion of a subject's cells, some amount of **Cryptosporidium** specific antibody may be detected in serum.

DETD Also provided herein is another **Cryptosporidium** diagnostic kit, that comprises anti-**Cryptosporidium** antibodies having specificity for one of the polypeptides of this invention; and instructions for use of the kit. Thus, kit may be utilized for the detection of **Cryptosporidium** peptides, a sign that there is parasite present in the subject being tested.

DETD **Cryptosporidium** parvum (AUCP-1) strain **oocysts** were obtained by infecting a 1 day old calf with 10.sup.6 **oocysts**. The calf was obtained at birth from the dairy herd at the Beltsville Agricultural Research Center and housed in a. . . the procedures described by Kilani et al. 1987. Am. J. Trop. Med. Hyg. 36: 505-508) for purification of C. parvum **oocysts**. Clean **oocysts** were resuspended in distilled water, stored at 4.degree. C., and used from 1-6 months after collection, depending on objectives of the experiment. Limited numbers of **oocysts** of other **Cryptosporidium** species were obtained from outside sources: C.

baileyi (B. Blagburn, Auburn University), C. meleagridis (M. Levy, North Carolina State University),. . .

DETD **Cryptosporidium oocysts** destined for RNA and DNA extraction were treated for 30 min. with 2.5% sodium hypochlorite (50% Clorox), washed 5 times. . . step was incorporated as per instructions from the manufacturer to remove polysaccharide which appears to exist in large quantities in **Cryptosporidium**. DNA of C. parvum was prepared by treating the parasite extract with 1% sodium dodecylsulfate (**SDS**) and 50 .mu.g/ml proteinase K (Gibco/BRL, Gaithersburg, Md.) as described (Jenkins et al. 1993. Infect. Immun. 61: 2377-2382). RNA and DNA yields were estimated by O.D..sub.260/O.D..sub.280 reading. Total **oocyst** protein was prepared by resuspending the parasites in protein extraction buffer (10 mM Tris-HCl pH 7.3, 1 mM MgCl.sub.2) in the presence of phenylmethylsulfonyl-fluoride (PMSF). The **oocysts** were subjected to five freeze-thaw cycles between dry ice-EtOH and 37.degree. C. water baths.

DETD **SDS**-PAGE/immunoblotting of Native and Recombinant C. parvum Protein

DETD Protein extracts of **Cryptosporidium oocysts** were treated with sample buffer containing 2-mercaptoethanol, heated for 3 min. in a **boiling** water bath, fractionated by 7.5-15% gradient **SDS**-PAGE, and transblotted to Immobilon (Millipore, Bedford, Mass.) membrane as described (Jenkins et al. 1993, supra). The antigen-impregnated membranes were treated. . .

DETD Identification of **Cryptosporidium** parvum-specific 41 kDa Protein

DETD Total **oocyst** protein extracted from C. parvum, C. baileyi, C. meleagridis, and C. serpentis was electrophoresed in adjacent lanes of a 7.5%-15% gradient **SDS**-PAGE (10.sup.7 **oocysts**/lane) and transblotted to nitrocellulose membrane (Schleicher and Schuell, Keene, N.H.). The blots were immunostained with rabbit antisera raised against total C. parvum **oocyst** protein to identify antigens unique to C. parvum.

DETD Antisera raised against total C. parvum **oocyst** protein recognized a number of antigens of C. parvum and C. baileyi (FIG. 1). A similar recognition pattern was observed with C. meleagridis and C. serpentis (data not shown). Although the majority of antigens were common to all **Cryptosporidium** species, a few antigens were unique to C. parvum. In particular, a 41 kDa protein was present in C. parvum but not in the other **Cryptosporidium** species (FIG. 1).

DETD Preparation of Antisera to **Cryptosporidium** parvum 41 kDa Protein

DETD Immunoscreening **Cryptosporidium** parvum Genomic DNA Libraries for p41 Clones

DETD The **Cryptosporidium** insert DNA was excised from pBluescript by PstI and HindIII digestion and ligated into the pTrcHis A vector (Invitrogen, Carlsbad,. . .

DETD Immunoblots of **SDS**-PAGE fractionated protein extracts from E. coli harboring recombinant pTrcHis-CP41 plasmid contained two unique protein bands, 28 and 36 kDa, produced. . .

DETD . . . shown). Antisera raised against native or recombinant CP41 antigen showed a similar pattern in immunoblots with whole native C. parvum **oocyst** protein (Cp), purified (P) or unpurified (IP) recombinant CP41 protein (FIG. 1). Normal control serum showed negligible recognition of native. . .

DETD . . . proteins did not appear to be due to glycan residues on the native protein. Glycosidase treatment was performed on total **oocyst** extracts to determine if CP41 was glycosylated. In addition, untreated **SDS**-PAGE-fractionated C. parvum protein impregnated on Immobilon membrane was treated with sodium perodiate to remove glycan residues. No difference in molecular. . .

DETD . . . and 28 kDa recombinant proteins may be due to the previously reported aberrant migration of E. coli-expressed recombinant proteins in **SDS**-PAGE for proteins with atypical amino acid compositions. The CP41 coding sequence contains a disproportionate number of Asn (21%) and

Thr. . .

DETD C. parvum sporozoites were first excysted by treating **oocysts** with 1% sodium hypochlorite (20% Clorox) for 10 min. at room temperature (RT), washing 5 times with Hank's balanced salt. . . for 1 hr in a humidified chamber at 37.degree. C. in 5% CO.sub.2. For examination by immunofluorescence microscopy, the excysted **oocyst**/sporozoite mixture was pipetted onto multi-well glass slides at 10.sup.4 **oocysts** per well (based on pre-excystation counts) and allowed to air dry at room temperature (RT). The parasites were then treated. . .

DETD The C. parvum **oocysts**/sporozoites were examined on an epifluorescence microscope. Antisera to native or recombinant CP41 antigen bound a surface antigen of C. parvum **oocysts** (FIG. 5). Although not evident from the IFA figures, the target antigen appeared to be distributed unevenly on the surface of the **oocyst**. Negligible staining of C. baileyi **oocysts** was observed with antisera to native or recombinant CP41 antigen (data not shown).

DETD For IEM, the excysted sporozoite/**oocyst** mixture was washed several times in PBS, enumerated on a hemocytometer, aliquoted at 10.sup.8 sporozoites into 1.5 ml microcentrifuge tubes, and pelleted by centrifugation. The **oocyst**/sporozoite pellet was resuspended in 2% paraformaldehyde, 0.5% glutaraldehyde in 0.1M sodium cacodylate for 20 min. at RT. The fixed parasites were washed twice with 0.1M sodium cacodylate and pelleted by centrifugation. The **oocyst** /sporozoite pellet was dehydrated in a graded ethanol series, infiltrated overnight in LR White hard grade acrylic resin, and cured at. . .

DETD . . . by IFA was confirmed by IEM (FIG. 6). The antigen was present on both external and internal regions of the **oocyst** wall and was also associated with amorphorous material on the **oocyst** outer surface.

DETD Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) Analysis of **Cryptosporidium** parvum **Oocyst** RNA

DETD C. parvum **oocysts** stored for 1, 3, or 6 mos. at 4.degree. C. were analyzed for the presence of CP41 messenger RNA using. . . To analyze for the presence of CP41 in genomic DNA, a single PCR was performed on DNA equivalent to 10.sup.3 **Cryptosporidium** **oocysts** using reaction conditions identical to those described above. The PCR products were analyzed by polyacrylamide gel electrophoresis, EtBr staining and. . .

DETD RT-PCR on total RNA showed that the CP41 transcript was present in C. parvum **oocysts** (FIG. 4). Although equivalent amounts of total RNA were used in each reaction, the intensity of the RT-PCR signal appeared to be inversely correlated with the age of C. parvum **oocysts**. The RT-PCR signal derived from RNA isolated from **oocysts** that had been stored at 4.degree. C. for 6 mo (CP1+) or 3 mo (CP2+) was about 25% and 50%, respectively, of the signal obtained from 1 mo old **oocysts** (CP3+, FIG. 3). The control reactions in which reverse transcriptase was not included did not show a PCR product indicating. . .

DETD . . . had experience sporadic outbreaks of cryptosporidiosis (natural infection). The calves were examined daily for diarrhea and excretion of C. parvum **oocysts** in feces using standard methods. Antisera was also obtained from a calf prior to and every week for one month after being fed NBC at birth and inoculated per os with 10.sup.6 C. parvum **oocysts** (experimental infection). Antisera were also obtained from adult cows (3-4 years old) before and for several weeks after experimental C.. . . cows that had not been exposed to C. parvum. Antisera were tested by ELISA for recognition of native C. parvum **oocyst** protein and recombinant CP41 antigen. In the former, C. parvum **oocysts** were subjected to multiple freeze-thaw cycles and disruption on a Mini-bead beater (Bespeak Products, Bartlesville, Okla.) followed by centrifugation to pellet insoluble material. The **oocyst** protein supernatant (50 .mu.l), equivalent to 4.times.10.sup.4 **oocysts** (150 ng protein), was pipetted onto individual wells of Immulon II microtiter plates and

incubated overnight at 4.degree. C. Native. . . by denaturing NiNTA affinity chromatography according to manufacturer's directions (Invitrogen). Eluates containing peak amounts of purified protein as indicated by **SDS**-PAGE/immunoblotting were pooled and adsorbed to the surface of Immulon II microtiter plates as described above. The wells were washed with. . .

DETD Recombinant CP41 antigen showed levels of binding similar to native C. parvum **oocyst** antigen when probed with sera from adult cows that were exposed to C. parvum (Table 1). The pre-infection titers to. . . and did not appear to increase appreciably after experimental challenge. Perhaps boosting of the antibody response was not observed because **oocyst** challenge did not result in patent infection due to the age-related resistance of adult cows to C. parvum infection. In. . . increase in Ab titers to rCP41 was noted after exposure to C. parvum. The Ab titers to native C. parvum **oocyst** antigen were variable. In two calves (FIGS. 7B,7D), the highest titers against native antigen occurred at time of **oocyst** shedding. In the other two calves, peak anti-C. parvum antigen titers were observed immediately after colostrum feeding (FIGS. 7A, 7C).. . . after an experimental C. parvum infection of a one-day-old calf (FIG. 7E). Antibody titers to rCP41 and native C. parvum **oocyst** antigen were highest after colostrum feeding and natural C. parvum **oocyst** inoculation (FIG. 7E). Although anti-rCP41 titers decreased with time, the response to native C. parvum **oocyst** titers was variable (FIG. 7E).

DETD

TABLE 1

Anti-recombinant Cp41 and native **Cryptosporidium** parvum **oocyst** antigen titers in sera from adult cows after experimental cryptosporidiosis infection or in unexposed control adult cows.

Time Post-infection Cow No.	(wk)	ELISA Titer rCP41	nCP **oocyst**
Experimental Infections:			
1	0	1100	2560
1	1	310	ND*
2	1	200	1280
3	1	200	2560
4	1	110	2560

. . .

DETD . . . hrs after the fusion. Hybridoma supernatants were screened by ELISA using rCP41 antigen and by immunofluorescence assay using C. parvum **oocysts** dried to the surface of multi-well glass slides. Cloning by limiting dilution was performed until a single clone was observed. . .

DETD

GENERAL INFORMATION:

NUMBER OF SEQ ID NOs: 4

SEQUENCE CHARACTERISTICS:

SEQ ID NO: 1

LENGTH: 740

TYPE: DNA

ORGANISM: **Cryptosporidium** parvum

SEQUENCE: 1

aatttcttct tttatgatga ttctaaaaag tatgagggag gattattaaa aaaagaaggt 60

tatgatggtt gtacagtagt tggtagtgat tgtttatgtt ggagatgtta tttcaatcaa 120

agaccatttt ttgaggagat ggactattct aggattccaa tttcttctga ggttatttgt 180

ggattattga. . . ataataataa taataatagt aataccactc ttactactgt tgctactaat 660

gctaatatta ctactaatac tactaatact actactacta ctactaataa taataataat 720

aataataata ataataattc 740

SEQUENCE CHARACTERISTICS:

SEQ ID NO: 2

LENGTH: 246
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 2
Asn Phe Phe Phe Tyr Asp Asp Ser Lys Lys Tyr Glu Gly Gly Leu Leu
1 5 10. . . Asn Asn Asn Asn Asn
225 230 235 240
Asn Asn Asn Asn Asn Asn
245
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 3
LENGTH: 20
TYPE: DNA
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 3
agcattagta gcaacagtag 20
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 4
LENGTH: 18
TYPE: DNA
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 4
gagatggact attctagg 18
CLM What is claimed is:
. . . sequence shown in SEQ ID NO:2 and wherein said protein is antigenic and effective to elicit an immune response against **Cryptosporidium** parvum.

3. A method of identifying **Cryptosporidium** parvum, said method comprising contacting a test sample suspected of containing **Cryptosporidium** parvum with an antibody that specifically and selectively binds rCP41 set forth in SEQ ID NO: 2 and detecting binding, wherein binding indicates the presence of **Cryptosporidium** parvum.

6. A method of identifying **Cryptosporidium** parvum antibodies in a subject, said method comprising contacting a test sample suspected of containing antibodies to **Cryptosporidium** parvum with an antigen wherein the antigen is rCP41 set forth in SEQ ID NO: 2 or native **Cryptosporidium** parvum CP41 and detecting antibody-antigen binding, wherein binding indicates the presence of **Cryptosporidium** parvum antibodies.

L4 ANSWER 2 OF 14 USPATFULL
ACCESSION NUMBER: 2003:33298 USPATFULL
TITLE: Methods for detection of Crytosporidium species and isolates and for diagnosis of **Cryptosporidium** infections
INVENTOR(S): Petersen, Carolyn, San Diego, CA, United States
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TI Methods for detection of Crytosporidium species and isolates and for diagnosis of **Cryptosporidium** infections

AB **Cryptosporidium** GP900, P68 and cryptopain antigens, antibodies, DNA or RNA for detection of **Cryptosporidium** in biological and environmental samples. A method for diagnosis of cryptosporidiosis. Kits and assays for the detection of **Cryptosporidium** comprising antigens, antibody, DNA or RNA components for immunological detection of **Cryptosporidium** protein with antibody, or detection of **Cryptosporidium** DNA by PCR amplification with GP900, P68 or cryptopain primers and probes for hybridization.

SUMM This invention concerns methods for detection of **Cryptosporidium** species or individual **Cryptosporidium** isolates and for diagnosis of prior or concurrent **Cryptosporidium** infections. The method for detection of **Cryptosporidium** species involves detection of a **Cryptosporidium** surface antigens GP900, p68 or cryptopain, GP900, p68 or cryptopain antibodies, and GP900, p68 or cryptopain DNAs and RNAs using PCR primers from regions flanking different domains of GP900, p68 or cryptopain. The method for detection of **Cryptosporidium** isolates involves PCR amplification of portions of GP900, P68, cryptopain and flanking regions with or without restriction fragment length polymorphism analysis which yield a fingerprint for each individual isolate. The method for diagnosis of **Cryptosporidium** infections involves detecting a presence of GP900, p68 or cryptopain antibodies, GP900, p68 or cryptopain antigen or the DNA or. . . RNA encoding the GP900, p68 or cryptopain antigen in biological and environmental samples. The methods of the invention detect the **Cryptosporidium** antigen comprised of a protein with or without carbohydrates attached thereto, or the DNA or RNA encoding the **Cryptosporidium** antigen or DNA adjacent to it (flanking regions), or a mutant, variant, analog or fragment thereof. The invention additionally concerns methods for production of recombinant **Cryptosporidium** antigens suitable for development of diagnostic and detection tools and kits.

SUMM **Cryptosporidium** is an Apicomlexan protozoa which causes gastrointestinal disease in humans and other vertebrates. In immunocompetent host, the disease process ends. . .

SUMM The genus **Cryptosporidium** consists of Apicomplexan parasites that invade and develop within epithelial cells of the gastrointestinal, hepatobiliary and respiratory tracts of a wide variety of vertebrates including reptiles, birds and mammals. **Cryptosporidium** was recognized as a cause of animal disease for several decades before the first cases of human cryptosporidiosis were reported. . . the magnitude of disease caused by this parasite in both AIDS patients and immunocompetent hosts began to be appreciated. Subsequently, **Cryptosporidium** has been found to be one of the most common causes of human diarrhea worldwide, and to be an increasingly recognized

cause of diarrhea in children, animal care workers, and travelers. (**Cryptosporidium** and Cryptosporidiosis in Humans, Ed. Fayer, R., CRC Press, Boca Raton (1997)).

SUMM Waterborne and nosocomial spread uncovered a number of biological characteristics of **oocysts**. First, the infectious dose of a parasite is very low. The ID50 for human volunteers with normal immune systems is 132 **oocysts** (N. Engl. J. Med., 332:855 (1995)). Second, infected hosts, for example calves, excrete large numbers of **oocysts**, on the order of 10.sup.10/day. Third, the **oocysts** are fully sporulated and ready to infect when excreted. Fourth, the **oocysts** are environmentally hardy. They remain infectious in cool, moist areas for 3-4 months and are not killed by chlorine levels achievable in drinking water. Fifth, the **oocysts** are quite small, 4-6 .mu.m, and are thus difficult to filter.

SUMM The infective forms of **Cryptosporidium**, called sporozoites and merozoites, appear to adhere to the host cell and release the contents of anterior organelles (rhoptries, micronemes. . .

SUMM While the actual interaction between **Cryptosporidium** and the host's immune system is poorly understood, it is known that disruption of either the cellular or the humoral. . . alone appear to be enough to neutralize the organism's infectivity. In vitro and in vivo observations indicate that antibodies to **Cryptosporidium** parvum inhibit invasion and intracellular development leading to protection in challenge experiments, or amelioration of infection in established disease (Infect.. . .

SUMM One source of such antibodies is hyperimmune bovine colostrum (HBC) collected from cows immunized with **Cryptosporidium** **oocysts**. Calves challenged with **Cryptosporidium** **oocysts** are protected from the development of disease by the administration of HBC (Infect. Immun., 61:4079 (1993)). Some immunocompromised AIDS patients infected with **Cryptosporidium** have also responded to HBC with a reduction in or disappearance of the symptoms of the disease (Gastroenterology, 98:486 (1990)).. . . and/or develop intracellularly in vitro and it has been used to immunoprecipitate at least 22 different surface radioiodinated proteins of **Cryptosporidium** sporozoites. Western blot analysis of proteins of whole **oocysts** which contain sporozoite indicates that HBC predominantly recognizes two proteins of sizes 250 KD and >900 KD (Infect. Immun., 61:4079. . .

SUMM Although a connection between **Cryptosporidium** from water, dairy animals, pets, children in day care and hospital environments and cryptosporidiosis has been made, the relative importance. . . food, pets, sexual or casual person-to-person contact in the transmission of the parasite has not been established. The epidemiology of **Cryptosporidium**, particularly transmission and reservoirs of the parasite have been difficult to study because the organism cannot be propagated in vitro for the development of serological or growth characteristics as a method of systematic identification of **Cryptosporidium** species and individual isolates.

SUMM Therefore, availability of **Cryptosporidium** specific antigen, DNA and antibody markers for species identification and for differentiation of strains/isolates within species would be greatly advantageous.

SUMM While, as described above, the **Cryptosporidium** infection can have serious and, in some cases, fatal consequences, only limited detection and diagnostic methods, tools and kits are. . .

SUMM Currently available **Cryptosporidium** detection methods are microbiological immunological assays and limited PCR-based detections. The microbiological detections of the organism in the stool has. . .

SUMM Thus, better and more sensitive methods for detection of **Cryptosporidium** are needed.

SUMM It is a primary objective of this invention to provide methods for detection of **Cryptosporidium** species or isolates by 1) specific detection of GP900, P68 or cryptopain antigens, 2) specific detection of GP900, P68 or cryptopain DNA fragments, and 3) detection of the presence of anti-**Cryptosporidium** antibodies indicating

that the host has been, in the past, or is currently infected with **Cryptosporidium**.

SUMM One aspect of this invention concerns methods and kits for detection of **Cryptosporidium** species or individual **Cryptosporidium** isolates and for diagnosis of prior or concurrent **Cryptosporidium** infections.

SUMM Another aspect of this invention concerns a method for detection of **Cryptosporidium** species involving detection of a **Cryptosporidium** antigen GP900, p68 or cryptopain, GP900, p68 or cryptopain antibodies, and GP900, p68 or cryptopain DNAs and RNAs using PCR. . .

SUMM Still another aspect of this invention concerns a method for detection of **Cryptosporidium** isolates involving PCR amplification of DNA fragments which are different in different isolates, such as poly-threonine rich domains or different. . .

SUMM Yet another aspect of this invention concerns a method for diagnosis of **Cryptosporidium** infections involving detection of the presence of GP900, p68 or cryptopain antibodies, GP900, p68 or cryptopain antigen or the DNA. . .

SUMM Still yet another aspect of this invention concerns methods for production of recombinant **Cryptosporidium** antigens suitable for development of diagnostic and detection tools and kits.

SUMM Another aspect of this invention concerns antibodies, antigens, DNAs and RNAs used in methods or kits for detection of **Cryptosporidium** species and isolates or diagnosis of prior or concurrent **Cryptosporidium** infections.

SUMM Another aspect of this invention concerns polyclonal or monoclonal antibodies directed against the **Cryptosporidium** antigen for use in a method and kits for detection of cryptosporidiosis.

SUMM Another aspect of this invention concerns the GP900, p68 or cryptopain **Cryptosporidium** antigen or fragments thereof.

SUMM Still another aspect of this invention concerns a DNA and RNA encoding the **Cryptosporidium** antigen and fragments thereof suitable for preparation of anti-**Cryptosporidium** antibodies.

SUMM . . . another aspect of the invention is the use of the GP900, p68 or cryptopain antigen, antibody, DNA or RNA for **Cryptosporidium** diagnosis of prior or current infection in a human or animal host or for detection of **Cryptosporidium** parasite in the environment.

SUMM Still another aspect of this invention concerns a DNA and RNA encoding the **Cryptosporidium** protein or glycoprotein comprising **Cryptosporidium** antigen or fragments thereof for use in production of the protein or glycoprotein for development of agents used for diagnosis or detection of **Cryptosporidium** infection.

SUMM Still yet another aspect of this invention concerns a method for diagnosing **Cryptosporidium** infection of a subject, comprising steps:

SUMM (a) contacting a body specimen, fluid or tissue obtained from the subject with an anti-**Cryptosporidium** monoclonal or polyclonal antibody; and

SUMM . . . detecting the formation of antibody-antigen complex wherein the presence of the complex indicates positive diagnosis of the presence of a **Cryptosporidium** organism in the subject and wherein the absence of the complex indicates negative diagnosis of **Cryptosporidium** infection.

SUMM Still yet another aspect of this invention concerns a method for detecting a presence of **Cryptosporidium** parasite in a biological or environmental sample, said method comprising steps:

SUMM (a) contacting a biological or environmental sample with an anti-**Cryptosporidium** monoclonal or polyclonal antibody; and

SUMM (b) detecting the formation of antibody-antigen complex wherein the presence of the complex indicates presence of the **Cryptosporidium** parasite in the sample and wherein the absence of the complex indicates absence of **Cryptosporidium** parasite in the sample.

SUMM Still yet another aspect of this invention concerns a method for detecting anti-**Cryptosporidium** antibody in a subject, said

method comprising steps:

SUMM . . . detecting a formation of antibody-antigen complex wherein the presence of the complex indicates positive diagnosis of the presence of a **Cryptosporidium** antibody in the subject and wherein the absence of the complex indicates negative diagnosis of **Cryptosporidium** infection.

SUMM Still another aspect of this invention is a **Cryptosporidium** diagnostic or detection kit comprising anti-**Cryptosporidium** specific monoclonal and polyclonal antibodies, **Cryptosporidium** GP900, P68 or cryptopain antigen according to the invention and a means for detection of an antibody-antigen complex.

DRWD FIG. 2 is an immunoblot of **Cryptosporidium** parvum **oocyst**/sporozoite proteins showing detection of the >900 sporozoite protein with monoclonal and polyclonal antibodies to GP900.

DRWD FIG. 3 is the immunoprecipitation of .sup.125I surface label **Cryptosporidium** parvum sporozoite proteins using monoclonal and polyclonal antibodies to GP900.

DRWD FIG. 5 is an immunoblot of **Cryptosporidium** parvum **oocyst**/sporozoite proteins identified by polyclonal antibodies to P68.

DRWD FIG. 9 is a genomic Southern analysis of **Cryptosporidium** DNA using the cryptopain probe.

DETD "**Cryptosporidium** species" means any organism belonging to the genus **Cryptosporidium**, such as, for example, **Cryptosporidium** parvum or **Cryptosporidium** muris, but also includes other currently less well characterized organisms such as, for example, Cyclospora. **Cryptosporidium** species comprise Apicomlexan parasites which primarily invade cells of the gastrointestinal tract and cause disease in a susceptible host.

DETD "**Cryptosporidium** isolate" means a viable **Cryptosporidium** separated from the other isolates having a specific genetic characterization (fingerprint) different from other isolates which may be identified by its specific genetic characterization and distinguished from other isolates having a different fingerprint. **Cryptosporidium** parvum was identified and isolated from **oocysts** of the Iowa, NINC and AUCP-1 isolates of **Cryptosporidium** parvum passaged through neonatal calves, as described in Example 1, and tested for their interaction with specific anti-GP900, p68 or. . .

DETD "Antigen" means a protein isolated from **Cryptosporidium** identified as GP900, P68 or cryptopain with or without glycoprotein attached thereto, detected in micronemes of developing merozoites and sporozoites.. . .

DETD "GP900 antigen" means a protein with or without a carbohydrate attached thereto which defines the capacity of **Cryptosporidium** sporozoites and merozoites to infect host cells. When deglycosylated, the GP900 core protein has a variable molecular weight of approximately. . . conditioned media. GP900 is the target of antibodies which are present and detectable in the tissue or fluids of the **Cryptosporidium** infected subject.

DETD . . . Mr between approximately 50 and 100 kilodaltons which is a target of antibodies which inhibit infection, invasion or adhesion of **Cryptosporidium**.

DETD . . . a protein which is a cathepsin L-like cysteine proteinase having a function in invasion and infection of host cells by **Cryptosporidium**. Cryptopain is represented by a protein containing 401 amino acids and is identified as SEQ ID NO: 30 (FIG. 3). . .

DETD "Detection" means establishing a priority evidence for the presence or prior presence of living or dead **Cryptosporidium** by detecting **Cryptosporidium** antigens, cryptopain or **Cryptosporidium** DNA or RNA in the host, in a host biological sample obtained from a specimen such as stool, urine, blood,. . .

DETD "Diagnosis" means establishment of the presence or prior presence of **Cryptosporidium** infection or disease by using the GP900, P68 or cryptopain antigen, antibody to GP900, P68 or cryptopain, GP900, P68 or.

. .

DETD . . . antibodies to GP900, P68 or cryptopain and structural variants described, such that the antibody inhibits infection, invasion or adhesion of **Cryptosporidium**.

DETD . . . The GP900, P68 or cryptopain protein has been identified as a target of anti-GP900, P68 or cryptopain antibodies which inhibit **Cryptosporidium** infection, invasion or adhesion.

DETD "Antibody-antigen complex" means a detectable complex of the anti-**Cryptosporidium** antibody bound to the **Cryptosporidium** antigen.

DETD . . . that cryptosporidiosis can be detected and diagnosed with a great sensitivity using methods and kits of the invention comprising specific **Cryptosporidium** antigens GP900, P68 and cryptopain, antibodies raised against these antigens, DNA or RNA probes or primers derived from DNA of. . .

DETD More specifically, the invention concerns detection of the presence of **Cryptosporidium** antigen or antibody for diagnosis of prior or current infection in humans and animals and for detection of **Cryptosporidium** in the environment.

DETD **Cryptosporidium** antigens described herein, in particular, are suitable for diagnosis of current or prior **Cryptosporidium** parvm infection by virtue of detecting antibodies to **Cryptosporidium**.

DETD Polyclonal or monoclonal antibodies to the **Cryptosporidium** antigens are suitable for detecting the presence of **Cryptosporidium** in biological samples of the host or in the environmental samples.

DETD The method for detection of the **Cryptosporidium** antigen presence in the biological or environmental samples comprises detecting antibody-antigen complex using specific monoclonal or polyclonal anti-**Cryptosporidium** antibodies to detect the presence of the antigen or detecting the presence of anti-**Cryptosporidium** antibody with **Cryptosporidium** antigen.

DETD This invention also provides DNA and RNA encoding the **Cryptosporidium** GP900, P68 or cryptopain molecule, or a mutant, variant, analog and fragment thereof, and methods for production of recombinant or. . .

DETD I. Detection of **Cryptosporidium** and Diagnosis of Cryptosporidiosis

DETD This invention primarily concerns methods and means for detection of **Cryptosporidium** in the biological and environmental samples or for diagnosis of prior or concurrent **Cryptosporidium** infection.

DETD A. Detection of **Cryptosporidium** in Biological and Environmental Samples

DETD The **Cryptosporidium** organism which causes cryptosporidiosis cannot be grown in vitro. This fact severely limits its detection in biological samples and in the environment because regularly used microbiological methods, such as culture, cannot be used for detection of fairly virulent **Cryptosporidium**.

DETD **Cryptosporidium** antigens GP900, P68 and cryptopain isolated from different **Cryptosporidium** species and isolates are differentiated by PCR amplification and/or restriction length polymorphism analysis which defines a fingerprint for each particular. . .

DETD Diagnosis of **Cryptosporidium** infection and detection of organisms in environmental samples uses the principle of hybridization of complimentary strands of DNA and RNA. . .

DETD . . . direct hybridization, a portion of a diagnostic sample or an environmental sample containing the target DNA or RNA, obtained from **Cryptosporidium**, is prepared for hybridization and denatured so that it interacts with and binds to a DNA probe. The DNA probe. . .

DETD . . . PCR primers or as hybridization probes for detection purposes. Any larger segment of DNA can be amplified by PCR from **Cryptosporidium** DNA and also used as a probe. In this invention all primers and probes which can be made from the DNA sequences of

GP900, P68 and cryptopain and used to detect **Cryptosporidium** DNA in diagnostic samples or environmental samples by way of direct hybridization or amplification methods are intended to be within. . .

DETD . . . the appropriate primers and conditions are used. With a small number of parasite and the specific primers, the presence of **Cryptosporidium** and fingerprinting of the isolate using environmental or biological sample permit using the method of the invention.

DETD . . . for environmental samples comprises contacting such a sample with the antigen or antibody of the invention for purposes of detecting **Cryptosporidium**. The detection method in biological samples comprises contacting a tissue body fluid, biopsy or solid specimen, with the antigen or antibody of the invention for purposes of detecting the presence of **Cryptosporidium**. Examples of body solid specimens are stool or tissue biopsies obtained from a subject. Examples of body fluids are blood,. . .

DETD Methods for actual detection of the presence of **Cryptosporidium** in the biological or environmental samples include but are not limited to polymerase chain reaction (PCR) amplification of the parasite. . .

DETD B. Detection of **Cryptosporidium** Isolates

DETD The invention also permits detection and identification of individual **Cryptosporidium** isolates based on their genetic characteristics, herein called "a fingerprint". Fingerprints from each individual isolate were discovered and are further. . .

DETD Differences between isolates of **Cryptosporidium** at the DNA level were previously described in J. Protozool 38: 405-415 (1991).

DETD . . . production of DNA probes which differentiate these isolates using a small amount of isolate DNA makes possible the detection of **Cryptosporidium** in water, food, pet, nosocomial and person-to-person nonsexual and sexual exposure. The development of isolate-specific **Cryptosporidium** probes also permits determination of a latent state of **Cryptosporidium** infection.

DETD Conserved PCR primers selected to amplify a polymorphic region of **Cryptosporidium** DNA from gastrointestinal tissue specimens of asymptomatic person will discover the presence of an organism which may later cause chronic. . .

DETD The description presented above describes the detection of DNA from **Cryptosporidium** in general and is general enough to detect all **Cryptosporidium** species and isolates.

DETD There are however, differences in sequences of different isolates of **Cryptosporidium** which may be exploited in order to detect and "thumbprint" different isolates. For example, the DNA of GP900 from 5. . .

DETD The detection of **Cryptosporidium** isolates depends on availability of conserved PCR primers which encompass polymorphic regions but which are conserved across C. parvum isolates. . .

DETD For the purposes of this invention, the following isolates of **Cryptosporidium** parvum were obtained: isolates which are geographically distinct (8); isolates which are temporally distinct (2), namely Iowa-1 and Iowa-2 isolates. . .

DETD An average of 5.times.10.sup.8 **oocysts** (2.times.10.sup.9 sporozoites) of each isolate was available in a largely purified form. 10.sup.9 **oocysts** of Iowa-2 was provided by Dr. L. Perryman. AUCP-1 and Iowa-1 isolates are available in >10.sup.9 **oocysts** quantities. SF1 and SF2 are available in semipurified form which has been roughly quantitated at >5.times.10.sup.8. Oocyts were purified and DNA isolated as previously described in J. Protozool., 38: 725-735 (1991). Using these techniques, 50-100 mg of **Cryptosporidium** DNA from 5.times.10.sup.8 **oocysts** were routinely isolated.

DETD For diagnostic detection of **Cryptosporidium** in a subject, the sample of the subject's tissue, body fluid or stool is obtained. The sample is then prepared to permit isolation of the parasite **oocyst**, for example by dissolving the stool (100 mg-3 g) in a buffer, such as TES buffer, centrifuging the suspension, separating the supernatant containing **oocysts** and recentrifuging to obtain **oocyst** pellet. After isolating the **oocysts**, the

oocysts are then submitted to lysing conditions where the **oocyst** releases DNA into the lysate. Lysate is then submitted to PCR DNA amplification with specific primers prepared as 15-35 bp. . . DNA is subjected to one of the detecting methods as described above, i.e., hybridization with the specific probe, immunodetection with **Cryptosporidium** specific antibody, with direct immunofluorescence, etc. In the alternative, the amplified DNA is applied on a column or another stationary. . .

DETD D. Kits for Detection of **Cryptosporidium**

DETD Kits for the diagnosis/detection of **Cryptosporidium** are used for determination of the presence or prior presence of the **Cryptosporidium** infection and environmental contamination, The kits comprise the antigen, antibody, DNA or RNA from GP900, P68 or cryptopain and a means for detecting the interaction of the materials of the invention with **Cryptosporidium** antigen, antibody, DNA or RNA in a host sample or **Cryptosporidium** antigen, DNA or RNA in an environmental sample.

DETD . . . utilized for the detection of endogenous antibodies/antigens/DNA/RNA produced by a subject that is afflicted with cryptosporidiosis or for detection of **Cryptosporidium** antigens/DNA/RNA present in the environmental samples. Due to the extreme sensitivity of the method of the invention, even at the. . . invasion of the subject's cells or when the parasite is extremely diluted, such as in water, some amount of the **Cryptosporidium** antigen DNA/RNA is detectable in stool or water samples using the kit of the invention. The kit may detect either 1) the antigen with the monoclonal or polyclonal antibodies; 2) the presence of the anti-**Cryptosporidium** antibody with the antigen; or 3) the presence of DNA/RNA by hybridization with DNA or RNA probe, PCR amplification with. . .

DETD II. **Cryptosporidium** Protein/Glycoprotein Antigens

DETD . . . parasitic disease for which there are currently no effective diagnostic or detection methods available. The current invention identifies several specific **Cryptosporidium** antigens and provide their DNA and amino acid sequences thereby permitting detection of the minute amount of the parasite DNA. . .

DETD During the development of this invention, it has been shown that a **Cryptosporidium** parvum comprises several highly pathogenic proteins which differ in their genetic make-up. These proteins were further identified as GP900, P68,. . .

DETD A. Identification of Protein GP900 as **Cryptosporidium** Antigen

DETD **Cryptosporidium** antigen identified as GP900 protein is a high molecular weight glycoprotein of a Mr greater than 900 kilodaltons (kD). The GP900 protein was detected in micronemes of developing merozoites and sporozoites of the invasive stages of **Cryptosporidium** by immunoelectronmicroscopy and has been shown to be accessible to surface radioiodination with .sup.125I. It is present on the surface. . .

DETD . . . complex. The GP900 protein was found to be highly abundant and easily visualized by Coomassie blue staining of proteins on **SDS** -polyacrylamide gels (**SDS**-PAGE) and is Triton X-100 soluble and N-glycosylated.

DETD GP900 proteins were identified and isolated from **oocysts** of the Iowa, AUCP-1, NINC or other isolates of **Cryptosporidium** parvum, as described in Example 1 and tested for their interaction with specific anti-GP900 antibodies. Proteins which were shown to. . .

DETD The GP900 gene of **Cryptosporidium** parvum was isolated from a naturally infected neonatal calf (NINC) isolate. DNA from the calf isolate was used to prepare. . .

DETD The presence of abundant cysteines on a surface protein of **Cryptosporidium** which is functionally homologous to the circumsporozoite protein of malaria strongly suggests that these cysteines participate in binding phenomena and. . .

DETD Recombinant GP900 protein useful in the method for diagnosis and detection of **Cryptosporidium** was cloned and expressed using methods described in Example 10.

DETD Sense and anti-sense PCR amplification oligonucleotides, which allowed

the amplification from Iowa genomic **Cryptosporidium** DNA of domain 1 or domain 3 with Kpn 1 and Xba I sequences at the 5' and 3' ends. . .

DETD Expressed portions of the GP900 loci are targets of polyclonal and monoclonal antibodies able to detect **Cryptosporidium** invasion. The expression, identification and isolation of these recombinant proteins allows production of recombinant proteins and antibodies to these proteins for the purpose of detection of **Cryptosporidium** in hosts or the environment and for diagnosis of prior or current **Cryptosporidium** infection in a suitable host.

DETD . . . of six antibodies, namely 10C6, 7B3, and E6, made from a single fusion event in which the immunogen was an **oocyst** containing sporozoites, were specific to GP900, suggesting that GP900 is a highly immunogenic molecule of sporozoites. Three of eight antibodies,. . .

DETD Polyclonal antibodies against **SDS** solubilized GP900 and MAb 10C6, prepared according to Example 4, which were previously shown to detect GP900, were used for detection of molecular species which are immunoprecipitable with both mono and polyclonal antibodies. A Western blot probe of **oocyst**/sporozoite proteins is seen in FIG. 2. Immunoprecipitation of sporozoite surface labeled proteins with mono and polyclonal antibodies as seen in. . .

DETD FIG. 2 shows an immunoblot of **Cryptosporidium** parvum **oocyst**/sporozoite proteins of the AUCP-1 isolate separated by **SDS**-PAGE. FIG. 2, lane 1 shows the MAb 10C6 culture supernatant, lane 2 shows the polyclonal anti-GP900 in 1:5000 dilution.

DETD FIG. 3 shows immunoprecipitation of .sup.125I radiolabelled **Cryptosporidium** parvum sporozoite surface proteins of the AUCP-1 isolate separated by 5-15% **SDS**-PAGE. FIG. 3, lane 1 shows radiolabelled **Cryptosporidium** parvum sporozoite surface protein control (10.sup.7 sporozoites/lane). Lane 2 shows radiolabelled **Cryptosporidium** parvum sporozoite surface proteins immunoprecipitated with polyclonal anti-GP900.

DETD . . . proteins were prepared according to Example 5. These various antibody preparations were used to probe an immunoblot of proteins from **Cryptosporidium** parvum **oocysts**/sporozoites as described in Example 7.

DETD Clearly, the clone S34 encodes a **Cryptosporidium** antigen and the antibodies specifically raised against this antigen are able to detect **Cryptosporidium** infection in vivo.

DETD Thus antibodies against the recombinant S34 protein are able to detect **Cryptosporidium** infection in vitro and in vivo indicating the usefulness of the anti-S34 antibody for both anti-**Cryptosporidium** detection and diagnosis of a human or animal host.

DETD A. Identification of Protein P68 as **Cryptosporidium** Antigen

DETD The second antigen protein, designated P68 was identified. The P68 **Cryptosporidium** antigen is a smaller protein. The P68 protein was partially sequenced at the DNA level. The 3' sequence and 3'. . .

DETD A **Cryptosporidium** antigen designated P68 is an apical protein of sporozoites and merozoites. The protein has a size of between about 50-100. . .

DETD . . . Immun., 60:5132-5138 (1992)). A recombinant eluted antibody from the clone identified a dominant 68 kDa protein on Western blot of **oocyst** sporozoite proteins and was localized to the anterior end of the sporozoite by indirect fluorescent antibody analysis.

DETD FIG. 5 is an immunoblot of AUCP isolate **oocyst**/sporozoite proteins. Lane 1 was detected with polyclonal anti-sporozoite/**oocyst** antibodies which had been affinity purified on the S19 fusion protein (S19-REA). As seen in FIG. 5, an immunoblot with. . .

DETD 3. Cryptopain--**Cryptosporidium** Parvum Antigen Cryptopain is cathepsin L-like cysteine proteinase.

DETD . . . were found to be suitable for and were therefore used to amplify a fragment of genomic DNA from Iowa isolate **Cryptosporidium** parvum **oocysts**.

DETD FIG. 9 is a genomic Southern analysis of **Cryptosporidium** DNA using the cryptopain probe. In FIG. 9, lane 1, the probe hybridizes to

two Hind III fragments. These fragments. . .

DETD . . . proteinase isolate are identical indicating that cryptopain is highly conserved in these isolates and that its function is essential for **Cryptosporidium**.

DETD SEQ ID NO: 88 is the DNA sequence of the **Cryptosporidium** cryptopain. The sequence (SEQ ID NO: 88) comprises 1663 base pairs and comprises 5' and 3' flanking sequences, pre, pro. . .

DETD Primers 7B1 and 7B2 were used to amplify the pre pro enzyme sequence from Iowa **Cryptosporidium** DNA. The primer 7B1 has a KpN1 site and the primer 7B2 has an XbaI site engineered into the 5'. . . expression of cryptopain and expression of the fusion protein, such as for example cryptopain-thioredoxin, at 57 kD, was analyzed by **SDS**-PAGE followed by immunoblot with antithioredoxin antibody.

DETD The current invention provides a method and a means for detection of the **Cryptosporidium** parasite and diagnosis of infection. The following examples describe procedures used to prepare the antigens and antibodies of the invention. . .

DETD **Cryptosporidium** parvum Parasites

DETD This example illustrates the protocol used for isolation of **Cryptosporidium** parvum parasites.

DETD **Oocysts** of the Iowa, NINC, AUCP-1 or other isolates of **Cryptosporidium** parvum described above were passaged through neonatal calves at the Animal Resources Services, University of California, Davis or obtained from a commercial source (Pat Mason) and the **oocysts** were purified and encysted. The detailed protocol is described in Infect. Immun., 61:4079 (1993). **Oocysts** containing sporozoites were solubilized, resolved by **SDS**-PAGE and subjected to immunoblotting, according to Infect. Immun., 60:5132 (1992).

DETD 10 week-old female BALB/c mice were immunized four times intraperitoneally with approximately 5.times.10.sup.5 sonicated 10.sup.5 **Cryptosporidium** parvum **oocysts**. The polyclonal antibody fraction of the ascites which was shown to react with the **Cryptosporidium** parvum sporozoite surface, the **oocyst** surface, and/or with internal antigens of the **oocysts**, was assessed by an IFA as described in Infect. Immun., 60:5132 (1992).

DETD For monoclonal antibody production, mice treated as above were immunized intravenously with the supernatant from sonicated **Cryptosporidium** parvum **oocysts** three days before fusion as described in J. Immunol., 123:1548 (1979) and J. Parasitol., 68:1029 (1982). Hybridoma supernatants were used. . .

DETD In order to determine whether GP900 was shed by the **Cryptosporidium** sporozoite in the absence of a specific antibody, living sporozoites were allowed to glide on poly-L-lysine coated microscopic slides. Slides. . .

DETD The Triton X-100 (1%) soluble fraction of 2.times.10.sup.8 **oocysts** was immunoprecipitated with MAb 10C6. A >900 kD MW species was identified in gels stained with Coomassie blue in water and excised. Frozen gel containing 2.times.10.sup.7 **oocyst** /sporozoites was pulverized and emulsified in 150 .mu.l PI of PBS and 150 .mu.l complete Freund's adjuvant (CFA) for intraperitoneal (IP). . .

DETD . . . in 200 .mu.l of 2M Tris, pH 7.4. All affinity purified antibodies reacted with the fusion protein and the respective **Cryptosporidium** protein but not other E. coli proteins.

DETD Polyclonal rabbit antisera from an unimmunized rabbit was evaluated for reactivity against **Cryptosporidium** antigens at a 1:1000 dilution on immunoblot and found to be free of reactivity. One ml of polyclonal rabbit antisera,. . . Tris, pH 6.0. Antibody concentration was determined by absorbance at 280 nm and integrity of the Ig was verified by **SDS**-PAGE. Positive control antibody, HBC Ig 40529 has been previously described in Infect. Immunol., 61:(10); 4079 (1993).

DETD **Oocysts** (10.sup.6 lane) were solubilized in denaturing sample buffer containing 5% .beta.ME (.beta.-mercaptoethanol), resolved by **SDS**-PAGE and subjected to immunoblotting according to Infect. Immunol., 60:532 (1992). Proteins were visualized after incubation with

primary antibody with .sup.125I. . .

DETD This example describes the Southern hybridization and Northern blot methods used for analysis of GP900 used for detection of **Cryptosporidium**.

DETD DNA was purified from 1.times.109 **Cryptosporidium** parvum **oocysts** as described in Example 1. DNA was digested with the restriction enzymes according to procedures provided by the manufacturer Promega.. . .

DETD mRNA was purified from MDCK cells, or MDCK cells infected with sporozoites at a ratio of 1 **oocyst**/1 MDCK cell, harvested at 24 and 48 hours using guanidinium thiocyanate and oligo-dT cellulose isolation (Ambion mRNA purification kit, Albion,. . .

DETD Surface Radioiodination and Immunoprecipitation of **Cryptosporidium** Sporozoite Proteins

DETD This example describes the methods used for surface radio-iodination and immunoprecipitation of **Cryptosporidium** sporozoite proteins.

DETD **Oocysts** were bleached, encysted and separated from sporozoites prior to iodination of the sporozoite surface and immunoprecipitation of surface proteins as. . .

DETD . . . 0.5% Triton X-100, pH 7.4) at 100,000.times.g for 1 hour at 40.degree. C. An aliquot of membrane proteins in 2% **SDS** 5% p-sample buffer was prepared for total sporozoite surface protein analysis. Aliquots of membrane proteins extracted in 2% **SDS** were diluted with 9 volumes NETT plus 1% high quality bovine serum albumin (BSA) obtained from Sigma; 1 volume 1%. . . overnight incubation. Protein A Sepharose 4B beads were added to immobilize the immunoprecipitated proteins. Parasite proteins were solubilized in 2% **SDS** sample buffer containing .beta.-mercaptoethanol. Samples were **boiled** 5 minutes and separated by 5-15% gradient **SDS**-PAGE.

DETD Iowa **oocysts** (5.times.10.sup.8) were excysted at 37.degree. C. for two hours and pelleted at 4,000.times.g for 10 minutes at 4.degree. C. The. . . 10 mM, PMSf 2 mM. The supernatant was concentrated by ultrafiltration to 350 .mu.l (14.2.times.) (Centricon 10, Amicon). Silver stained **SDS**-PAGE gel of 10 and 20 .mu.l aliquots revealed equal amounts of 47 kD, 120 kD and >900 kD proteins. The. . .

DETD . . . the domain 1 sequence. When used as a pair of PCR amplification oligonucleotides, these oligonucleotides allowed the amplification from genomic **Cryptosporidium** DNA of the entire domain 1 and domain 3 sequences with Kpn 1 and Xba I sequences at the 5'. . .

DETD Detection of **cryptosporidium** in Stool Samples Using Monoclonal Anti-**Cryptosporidium** Antibody

DETD This example describes immunodetection of **Cryptosporidium** in stool samples.

DETD . . . and allowed to air dry. The slide was incubated with a fluorescein isothiocyanate-labeled monoclonal antibody M10 or 10C6 to GP900 **Cryptosporidium oocysts** antigen in a modified kit purchased from Meridian Diagnostics, Cincinnati. Positive and negative control reagents supplied with the kits were included in each test. **Oocysts** in 10.times.40 fields (2.5% of the sample area) were counted by fluorescence microscopy. If no organisms were seen, the entire slide was examined using a .times.20 objective, and all **oocysts** were counted.

DETD . . . assayed on 3 separate days. The mean of triplicate counts in the 5-.mu.L aliquot was standardized to the number of **oocysts** per milliliter of stool, taking into account the 1:4 dilution in preservative. Total **oocyst** excretion per specimen was calculated by multiplying the mean concentration (**oocysts** /milliliter) by stool weight (grams). Total daily **oocyst** excretion was the sum of **oocysts** in each specimen in a 24-h period.

DETD Detection of **Cryptosporidium** in Biopsy

DETD This example describes the procedure for detection of **Cryptosporidium** in tissue biopsy samples.

DETD PCR Assay for Detection of **Cryptosporidium** DNA in Feces

DETD This example describes conditions for PCR assay for detection of DNA

from **Cryptosporidium**.

DETD DNA extraction of **oocyst** from stool was performed as follows. One-gram portions of fecal material were mixed with 40 ml of 0.35% sodium hydrochloride. . . was filtered through a 3-.mu.m-millipore filter. The filter was washed with 3 ml Tris HCl (pH 8). Recovery of the **oocysts** was completed by shaking the filters for 30 seconds in the tubes with Tris HCl. After discarding the filters, the. . .

DETD The PCR amplification of **Cryptosporidium** Iowa isolate domain 3 was performed as follows. For each sample analyzed, 10 .mu.l of lysate was used as a. . .

DETD This example describes the techniques used for detection of **Cryptosporidium** in stool samples using specific anti-GP900 antibodies.

DETD Fresh fecal specimens were obtained for detection of **Cryptosporidium** antibodies. Fecal samples were diluted one in three (weight by volume) with distilled water and thoroughly mixed using a vortex. . .

DETD . . . M2 or M10. Examination was performed at .times.400 magnification under bright-field microscopy, and fluorescence microscopy. Total and average numbers of **oocysts** were recorded for every ten fields in each smear. Negative control smears were prepared from samples which were not inoculated with **oocysts**.

DETD . . . colony hybridization procedure was performed and filters were screened with .sup.32P-labeled, genomic Cyclosporidium DNA obtained from GP900, P68 or cryptopain **Cryptosporidium** antigens. Duplicate filters were screened by the same method using genomic DNA from G. lamblia, P. falciparum, Toxoplasma gondii, and. . .

DETD Polymerase Chain Reaction for Detection of **Cryptosporidium** Isolates

DETD This example describes PCR amplification procedure used for detection of **Cryptosporidium** isolates.

DETD . . . 5 min, precipitated with 0.4 M LiCl and ethanol, vacuum-dried, and resuspended in 20 .mu.l of TE buffer with 0.1% **SDS**. Molecular size markers electrophoresed with the PCR samples consisted of a Hind III digest of .lambda. DNA and a Hae. . .

DETD Membranes were prehybridized for 1 hr at 42.degree. C. in 5.times.SSC, 5% (w/v) **SDS**, and 50% (v/v) formamide (hybridization solution). After prehybridization, the membranes were transferred to heat-sealable bags. Approximately 2 ml of hybridization. . . C. After hybridization, the membranes were washed twice (5 min/wash) at room temperature in 50 ml of 20.times.SSC, 0.1% 9w/v) **SDS**, followed by two washes (15 min/wash) in 0.1% (w/v) **SDS** at 50.degree. C. Prior to antibody labeling, the membranes were equilibrated for 1 min at room temperature in 100 mM. . .

DETD

GENERAL INFORMATION:

NUMBER OF SEQ ID NOs: 115

SEQUENCE CHARACTERISTICS:

SEQ ID NO: 1

LENGTH: 7334

TYPE: DNA

ORGANISM: **Cryptosporidium** parvum

SEQUENCE: 1

gatcctgcaa tgtggcaaat ggttacaact atagcagcaa tttgtagtac tgcatgccag 60

aatggtggta gaagtagtag acattgctgt agaaaacatc attctagaaa gcataaaaaa 120

gaagcagaat taaaagatac taatagcaat agcagtaaca aggaatcgag cgaccttagt 180

gaagctcaaa. . . tactacattt ctacatttcc tattgaaata tacgatttac taacatattg 7260

ctaattaata aatgattaat aatgacaaaa ttcaacgata tgatgaatct atcaaagcgt 7320

ttcaaatgga gaaa 7334

SEQUENCE CHARACTERISTICS:

SEQ ID NO: 2

LENGTH: 5511

TYPE: DNA

ORGANISM: **Cryptosporidium** parvum

SEQUENCE: 2

gtgaagtcaa aaatcatggt gaacattaaa gtgagctcat cggcaatagc ccttgtggct 60
gttattatga acccactttt ttcacttgca tttaaatcga gtaaccgatt agagatgaga 120
attgaatcat ctggtgcagt ttcaaatgaa aaatttgtaa tcccatctct cccttcagat 180
ttagacccaa. . . gaggaagaag 5400
agggtgatga agcagcaaat gaaactgttg ttacaattga gcgtgattca tcattctgga 5460
acgaatctta aacgtagaaa agatttttcc aattcaaaaa aatttcgaat a 5511
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 3
LENGTH: 5318
TYPE: DNA
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 3
attttggaag gttcaattgc aggtattaga agcgaatctt gcattgtatc tgaactgaac 60
tttacatcta ctactggatt tacaacggac acatcaatga attggccggt aagtatcaca 120
agtggtgaac tgaaggatcc aaacaaacaa gctactattt ctggttcaag atcttgtgga 180
tggaaacaag. . . atattactac atttctacat ttcctattga 5220
aatatacgat ttactaacat attgctaatt aataaatgat taataatgac aaaattcaac 5280
gatatgatga atctatcaaa gcgtttcaaa tggagaaa 5318
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 4
LENGTH: 5163
TYPE: DNA
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 4
attttggaag gttcaattgc aggtattaga agcgaatctt gcattgtatc tgaactgaac 60
tttacatcta ctactggatt tacaacggac acatcaatga attggccggt aagtatcaca 120
agtggtgaac tgaaggatcc aaacaaacaa gctactattt ctggttcaag atcttgtgga 180
tggaaacaag. . .
DETD . . . 5040
aagaggaaga agagggtgat gaagcagcaa atgaaactgt tgttacaatt gagcgtgatt 5100
catcattctg gaacgaatct taaacgtaga aaagattttt ccaattcaaa aaaatttcga 5160
ata 5163
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 5
LENGTH: 1837
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 5
Val Lys Ser Lys Ile Met Val Asn Ile Lys Val Ser Ser Ser Ala Ile
1 5 10. . . Arg Lys Asp Phe Ser Asn Ser Lys
Lys Phe Arg Ile
1825 1830 1835
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 6
LENGTH: 1721
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 6
Ile Leu Glu Gly Ser Ile Ala Gly Ile Arg Ser Glu Ser Cys Ile Val
1 5 10. . .
DETD . . . Asp
1700 1705 1710
Phe Ser Asn Ser Lys Lys Phe Arg Ile
1715 1720
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 7
LENGTH: 303
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 7
Met Val Asn Ile Lys Val Ser Ser Ser Ala Ile Ala Leu Val Ala Val
1 5 10. . . Ile Pro Tyr Thr Lys Cys Val Gly
Val Lys His
290 295 300
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 8

LENGTH: 216
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 8
Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr Thr
1 5 10. . . Thr Thr
195 200 205
Thr Ala Thr Thr Thr Thr Thr Thr
210 215
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 9
LENGTH: 159
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 9
Ser Glu Thr Glu Ser Val Ile Lys Pro Asp Glu Trp Cys Trp Leu Glu
1 5 10. . . Ile His Ser Gly Tyr Gln Thr Ser
Ala Asp Phe Val
145 150 155
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 10
LENGTH: 112
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 10
Thr Thr Thr Thr Ala Lys Pro Thr Thr Thr Thr Thr Gly Ala Pro Gly
1 5 10. . . Thr Lys Arg Asp Glu Met Thr Thr
Thr Thr Thr
100 105 110
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 11
LENGTH: 1042
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 11
Pro Leu Pro Asp Ile Gly Asp Ile Glu Ile Thr Pro Ile Pro Ile Glu
1 5 10. . . Arg Lys Asp Phe Ser Asn Ser Lys
Lys Phe
1025 1030 1035 1040
Arg Ile
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 12
LENGTH: 128
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 12
Met Gly Ser Lys Val Tyr Ile Pro Tyr Thr Lys Cys Val Gly Val Lys
1 5 10. . .
DETD . . . Val Ile Lys Pro Asp Glu Trp Cys Trp Leu Glu
115 120 125
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 13
LENGTH: 130
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 13
Met Gly Ser Lys Val Tyr Ile Pro Tyr Thr Lys Cys Val Gly Val Lys
1 5 10. . . Val Ile Lys Pro Asp Glu Trp Cys
Trp
115 120 125
Leu Glu
130
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 14
LENGTH: 130
TYPE: PRT

ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 14
Met Gly Ser Lys Val Tyr Ile Pro Tyr Thr Lys Cys Val Gly Val Lys
1 5 10. . . Val Ile Lys Pro Asp Glu Trp Cys
Trp
115 120 125
Leu Glu
130
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 15
LENGTH: 138
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 15
Met Gly Ser Lys Val Tyr Ile Pro Tyr Thr Lys Cys Val Gly Val Lys
1 5 10. . . 115 120
125
Ile Lys Pro Asp Glu Trp Cys Trp Leu Glu
130 135
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 16
LENGTH: 124
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 16
Met Gly Ser Lys Val Tyr Ile Pro Tyr Thr Lys Cys Val Gly Val Lys
1 5 10. . . 110
Ser Val Ile Lys Pro Asp Glu Trp Cys Trp Leu Glu
115 120
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 17
LENGTH: 175
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 17
Met Gly Ser Lys Val Tyr Ile Pro Tyr Thr Lys Cys Val Gly Val Lys
1 5 10. . . Val Ile Lys Pro Asp Glu Trp Cys
Trp Leu Glu
165 170 175
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 18
LENGTH: 150
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 18
Met Gly Ser Lys Val Tyr Ile Pro Tyr Thr Lys Cys Val Gly Val Lys
1 5 10. . . Val Ile Lys Pro Asp
130 135 140
Glu Trp Cys Trp Leu Glu
145 150
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 19
LENGTH: 91
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 19
Met Gly Ser Lys Val Tyr Ile Pro Tyr Thr Lys Cys Val Gly Val Lys
1 5 10. . . 75 80
Val Ile Lys Pro Asp Glu Trp Cys Trp Leu Glu
85 90
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 20
LENGTH: 249
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 20

Met Gly Ser Lys Val Tyr Ile Pro Tyr Thr Lys Cys Val Gly Val Lys
1 5 10. . . 63

LENGTH: 20
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence Primer
SEQUENCE: 63
cactgaaata tttaccagag 20

SEQUENCE CHARACTERISTICS:
SEQ ID NO: 64
LENGTH: 1509
TYPE: DNA
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 64
agtaagggtc aattatttaa cccagtaagt aagttgtgtg tacgacttaa agacaatgtt 60
gtaggtggag gagctctggt tttggatgat tgtcgtaaag ctagtgatgg aagtggatta 120
ttcgaattaa tgccaaacaa tcagctcaga ttagctagag gtggaaatct atgcttaaca 180
agtccaggag. . . 1380
aactctacat ctggagcact tccagaactg gttctttgca gtacaaatac caatttgaag 1440
catgaaagca atgcaatttc cttgtcttgt gaaagcagat tctctgatat gaaggtattt 1500
catttggat 1509

SEQUENCE CHARACTERISTICS:
SEQ ID NO: 65
LENGTH: 2380
TYPE: DNA
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 65
agtaagggtc aattatttaa cccagtaagt aagttgtgtg tacgacttaa agacaatgtt 60
gtaggtggag gagctctggt tttggatgat tgtcgtaaag ctagtgatgg aagtggatta 120
ttcgaattaa tgccaaacaa tcagctcaga ttagctagag gtggaaatct atgcttaaca 180
agtccaggag. . .
DETD . . . tcttctccgt gatactttca gtaggatttg 2280
atgcgcgtaa atacagggct ttcctgcttg ttgaaatggc cagtttctgt aatttgagtt 2340
ttttcctcac tttcagactg ttctggataa tccggaattt 2380

SEQUENCE CHARACTERISTICS:
SEQ ID NO: 66
LENGTH: 503
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 66
Ser Lys Gly Gln Leu Phe Asn Pro Val Ser Lys Leu Cys Val Arg Leu
1 5 10. . . 87

LENGTH: 20
TYPE: DNA
ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence Primer
SEQUENCE: 87
agtaggattt gatgcgcgta 20

SEQUENCE CHARACTERISTICS:
SEQ ID NO: 88
LENGTH: 1663
TYPE: DNA
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 88
caaaacttcc taatttctca atgtattact aattaataga aagtttgttt tattttcatg 60
tggataaatg aattattttc tctataccgg catttgcatg caattttgta tgactaaaat 120
gtaaataatt atttgcatgc aattatgtgg gcatgtcata gtttttcaag aataataata 180
agatgacatg. . . tattcatata tataagcatt 1560
ccatacttaa ttatttattg attttaataa aatgtttggc taaagaaagc aatcaagata 1620
atttatggac gttctattgt tcttacttca ataataatcc ttt 1663

SEQUENCE CHARACTERISTICS:
SEQ ID NO: 89
LENGTH: 534
TYPE: DNA

ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 89
ttaagtaaaa tggacatagg aaacaacgtg gaagaacatc aggaatatat ttctggacca 60
tacattgcat taattaatgg cactaatcaa caaagggaac cgaataaaaa gttgaaaaac 120
ataataattg caacgttgat tgcaatcttt atagttttgg ttgttactgt atctttgtat 180
attactaata. . . tggtgatttg 420
tcgaaagaag agtttatggc aagattcaca ggatatataa aagattccaa agatgatgaa 480
agggtattta agtcaagtag agtctcagca agcgaatcag aagaggaatt tgtt 534
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 90
LENGTH: 678
TYPE: DNA
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 90
cccccaaatt ctattaattg ggtggaagct ggatgcgtga acccaataag aaatcaaaag 60
aattgtgggt catgttgggc tttctctgct gttgcagctt tggagggagc aacgtgtgct 120
caaacaaacc gaggattacc aagcttgagt gaacagcaat ttgttgattg cagtaaacaa 180
aatggcaact. . . tagtaagaaa tagctggggt gaagcgtggg gagagaaagg atacatcaaa
600
ctagctcttc attctggaaa gaagggaaca tgtggtatat tggttgagcc agtgtatcca 660
gtgattaatc aatcaata 678
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 91
LENGTH: 401
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 91
Met Asp Ile Gly Asn Asn Val Glu Glu His Gln Glu Tyr Ile Ser Gly
1 5 10. . . Val Glu Pro Val Tyr Pro Val Ile
Asn Gln Ser
385 390 395 400
Ile
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 92
LENGTH: 175
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 92
Met Asp Ile Gly Asn Asn Val Glu Glu His Gln Glu Tyr Ile Ser Gly
1 5 10. . . Val Ser Ala Ser Glu Ser Glu Glu
Glu Phe Val
165 170 175
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 93
LENGTH: 226
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 93
Pro Pro Asn Ser Ile Asn Trp Val Glu Ala Gly Cys Val Asn Pro Ile
1 5 10. . . Val Glu Pro Val Tyr Pro Val Ile
Asn Gln
210 215 220
Ser Ile
225
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 94
LENGTH: 457
TYPE: DNA
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 94
tgctgttgca gctttggagg gagcaacgtg tgctcaaaca aaccgaggat taccaagctt 60
gagtgaacag caatttgttg attgcagtaa acaaaatggc aactttggat gtgatggagg 120
aacaatggga ttggcttttc agtatgcaat taagaacaaa tatttatgta ctaatgatga 180
ttacccttac. . .
DETD . . . 23
TYPE: DNA

ORGANISM: Artificial Sequence
FEATURE:
OTHER INFORMATION: Description of Artificial Sequence Primer
SEQUENCE: 110
cctctagatg cttatattga ttg 23
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 111
LENGTH: 7
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 111
Cys Gly Ser Cys Trp Ala Phe
1 5
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 112
LENGTH: 8
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
FEATURE:
NAME/KEY: MOD_RES
LOCATION: (3)
OTHER INFORMATION: L or I
SEQUENCE: 112
Tyr Trp Xaa Val Arg Asn Ser Trp
1 5
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 113
LENGTH: 5
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 113
Val Arg Asn Ser Trp
1 5
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 114
LENGTH: 1203
TYPE: DNA
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 114
atggacatag gaaacaacgt ggaagaacat caggaatata tttctggacc atacattgca 60
ttaattaatg gcactaatca acaaagggaa ccgaataaaa agttgaaaaa cataataatt 120
gcaacgttga ttgcaatctt tatagttttg gttgttactg tatctttgta tattactaat 180
aacaccagtg. . . 1080
agaaatagct ggggtgaagc gtggggagag aaaggataca tcaaactagc tcttcattct 1140
ggaaagaagg gaacatgtgg tatattggtt gagccagtgt atccagtgat taatcaatca 1200
ata 1203
SEQUENCE CHARACTERISTICS:
SEQ ID NO: 115
LENGTH: 540
TYPE: PRT
ORGANISM: **Cryptosporidium** parvum
SEQUENCE: 115
Gln Asn Phe Leu Ile Ser Gln Cys Ile Thr Asn Lys Val Cys Phe Ile
1 5 10. . .
CLM What is claimed is:
1. A method for detection of a **Cryptosporidium** species or isolate in a biological and environmental sample, said method comprising steps: a) obtaining the biological or environmental sample; b) contacting the sample of step (a) with an **Cryptosporidium** GP900, P68 or cryptopain antigen, DNA or RNA or with a specific anti-**Cryptosporidium** GP900, P68 or cryptopain antibody, wherein said GP900 antigen is a protein comprising 1832 amino acids depicted by SEQ ID. . . presence of said antigen-antibody complex, or a presence of DNA or RNA indicates a presence and a positive detection of **Cryptosporidium** in the sample and an absence of such complex, or absence of DNA or RNA indicates an absence of **Cryptosporidium**.

3. The method of claim 2 wherein the **Cryptosporidium** is detected immunologically.

9. The method of claim 1 wherein the **Cryptosporidium** GP900, P68 or cryptopain antigen is detected with polymerase chain reaction (PCR) or by hybridization.

. . . 10. The method of claim 9 wherein the PCR or hybridization is performed on the sample pretreated to release a **Cryptosporidium oocyst** DNA.

. . . with DNA and wherein PCR comprises an amplification of a specific nucleic acid sequence comprised within DNA released from the **Cryptosporidium oocyst**.

15. The method of claim 11 wherein the **Cryptosporidium** DNA is detected by the direct hybridization.

. . . method of claim 21 wherein said detection of GP900, P68 and cryptopain species or isolates is used for diagnosis of **Cryptosporidium** infections in human or animal tissue samples.

23. A kit for detection of **Cryptosporidium** species or isolate in a biological or environmental sample consisting essentially of: a means for contacting said sample with one of the following: a recombinant **Cryptosporidium** GP900 antigen selected from a group consisting of a protein depicted by the SEQ ID NO: 5-20; a recombinant P68. . . the group consisting of a protein depicted by the SEQ ID NOs: 91-93, and 111-113; for detection of a specific anti-**Cryptosporidium** GP900, anti-P68 or anti-cryptopain antibody; or an anti-**Cryptosporidium** GP900 antibody raised against a **Cryptosporidium** GP900 antigen selected from a group consisting of a protein depicted by the SEQ ID NO: 5-20 for detection of the GP900 antigen; or an anti-P68 antibody raised against a **Cryptosporidium** P68 antigen selected from a group consisting of a protein depicted by the SEQ ID NO: 66; or an anti-cryptopain. . . consisting of a protein depicted by the SEQ ID NO: 91-93 and 111-113; for detection of a presence of a **Cryptosporidium** GP900, P68 or cryptopain antigen; and a means for detection of a reaction between the antigen and antibody.

. . . to detect the presence of the anti-GP900, anti-P68 or anti-cryptopain antibody or wherein the means for contacting sample is the anti-**Cryptosporidium** antibody and said antibody is used to detect the presence of the antigen.

29. A kit for detection of **Cryptosporidium** in a biological or environmental sample consisting essentially of: a means for contacting said sample with an oligonucleotide probe for. . . (PCR) amplification and detection of a nucleotide sequence depicted ID SEQ NOs: 1-4, 64, 65, 88-90, 94 and 114 of **Cryptosporidium** GP900, P68 or cryptopain DNA or RNA; and a means for detection of a reaction between the probe and the. . .

. . . the sample is the oligonucleotide probe for hybridization with DNA or RNA, wherein said probe comprises oligonucleotide sequences complementary to **Cryptosporidium** GP900, P68 or cryptopain DNA or RNA.

. . . DNA or RNA comprising an oligonucleotide sequence of about 14 to about 35 bp of the genomic DNA sequence of **Cryptosporidium** GP900, P68 or cryptopain DNA depicted by SEQ ID NOs: 1-4, 25 or 88-90.

L4 ANSWER 3 OF 14 USPATFULL
ACCESSION NUMBER: 2002:295323 USPATFULL
TITLE: Recombinant neospora antigens and their uses

INVENTOR(S):	Conrad, Patricia C., Davis, CA, UNITED STATES Barr, Bradd C., Davis, CA, UNITED STATES Anderson, Mark L., Davis, CA, UNITED STATES Sverlow, Karen W., Vacaville, CA, UNITED STATES
PATENT ASSIGNEE(S):	THE REGENTS OF THE UNIVERSITY OF CALIFORNIA, Oakland, CA, UNITED STATES, 94607-5200 (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 2002165373	A1	20021107
APPLICATION INFO.:	US 2001-957995	A1	20010921 (9)

RELATED APPLN. INFO.:	Continuation of Ser. No. US 1999-281766, filed on 30 Mar 1999, GRANTED, Pat. No. US 6376196 Continuation of Ser. No. US 1996-645951, filed on 10 May 1996, GRANTED, Pat. No. US 5889166 Continuation of Ser. No. US 1994-327516, filed on 20 Oct 1994, GRANTED, Pat. No. US 5707617 Continuation of Ser. No. US 1994-215858, filed on 21 Mar 1994, ABANDONED
DOCUMENT TYPE:	Utility
FILE SEGMENT:	APPLICATION
LEGAL REPRESENTATIVE:	TOWNSEND AND TOWNSEND AND CREW, LLP, TWO EMBARCADERO CENTER, EIGHTH FLOOR, SAN FRANCISCO, CA, 94111-3834
NUMBER OF CLAIMS:	27
EXEMPLARY CLAIM:	1
NUMBER OF DRAWINGS:	3 Drawing Page(s)
LINE COUNT:	2670

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

DETD . . . to the sequences for this gene in other coccidial parasites. Alignment of these sequences with published sequences of Neospora caninum, **Cryptosporidium** parvum, Sarcocystis muris and Toxoplasma gondii showed that the bovine Neospora isolate is genotypically unique.

DETD . . . biological samples. These sequences can be used to detect all stages of the Neospora life cycle (e.g., tachyzoites, bradyzoites, and **oocysts**) in biological samples from both the bovine host and the definitive host. A variety of methods of specific DNA and. . .

DETD . . . are particularly useful for the diagnosis of neosporosis and identification of the source of Neospora parasite stages (tachyzoites, bradyzoites and **oocysts**) in various animal hosts.

DETD . . . Neospora. The compositions of the invention can also be used to treat the definitive host to prevent the shedding of **oocysts** and subsequent transfer to cattle. The compositions for administration to either cattle or the definitive host can comprise tachyzoite and/or. . .

DETD . . . 4 different antisera to T. gondii (Tg1-4). Antiserum Tg1 was produced by the infection of a rabbit with live sporulated **oocysts** of the ME-49 strain (Lindsay & Dubey, supra) of T. gondii and used at a 1:400 dilution. Toxoplasma gondii antiserum. . .

DETD . . . antigen expression of the different parasites and variations in the methods used to produce the antisera (i.e. immunization with cysts, **oocysts** or tachyzoite lysates). For example, tissue cyst wall antigens that reacted with antiserum to H. hammondi appeared to be lacking. . .

DETD . . . DNA was prepared as follows. Briefly, the parasite or control cell pellets were suspended in 1.0 ml STE with 0.5% **SDS** treated with proteinase. K (100 .mu.g/mL) and RNAase (100 .mu.g/mL) then extracted twice with phenol, once with phenol-chloroform-isoamyl alcohol, and. . .

DETD . . . After hybridization, the membranes were washed twice for 5 min each at room temperature in 5X SSC and 0.1% (w/v) **SDS**, and then washed twice for 5 min each at room temperature in 0.5X SSC and 0.1% (w/v) **SDS**. Membrane blocking, antibody incubations, signal generation and detection were performed as described by the manufacturer. Membranes were exposed to Kodak. . .

DETD . . . with the probes overnight at 42.degree. C. The blots were washed at 42.degree. C. (2.times.20 min.) in 6M Urea, 0.4% **SDS**

, 0.5 x SSC and rinsed with 20.times.SSC prior to autoradiography.

DETD . . . when the Eco R1 insert from clone N54 was used as a probe, the wash protocol was adjusted to 0.4% **SDS**, 0.5.times.SSC at 45EC (2.times.20 min.).

DETD . . . Both recombinant antigens and parasite antigens were quantitated with the BCA Protein Assay (Pierce), denatured in Laemmli's sample buffer and **boiled** for 5 minutes prior to electrophoresis. **SDS**-PAGE and Western blots were performed under standard conditions.

L4 ANSWER 4 OF 14 USPATFULL

ACCESSION NUMBER:	2002:88219 USPATFULL
TITLE:	Recombinant neospora antigens and their uses
INVENTOR(S):	Conrad, Patricia, Woodland, CA, United States Louie, Kitland, San Francisco, CA, United States
PATENT ASSIGNEE(S):	The Regents of the University of California, Oakland, CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6376196	B1	20020423
APPLICATION INFO.:	US 1999-281766		19990330 (9)
RELATED APPLN. INFO.:	Continuation-in-part of Ser. No. US 1996-645951, filed on 10 May 1996, now patented, Pat. No. US 5889166		

DOCUMENT TYPE:	Utility
FILE SEGMENT:	GRANTED
PRIMARY EXAMINER:	Navarro, Mark
LEGAL REPRESENTATIVE:	Townsend and Townsend and Crew LLP, Bastian, Kevin L., Wang, Hugh
NUMBER OF CLAIMS:	7
EXEMPLARY CLAIM:	1
NUMBER OF DRAWINGS:	6 Drawing Figure(s); 3 Drawing Page(s)
LINE COUNT:	2125

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

DETD . . . to the sequences for this gene in other coccidial parasites. Alignment of these sequences with published sequences of Neospora caninum, **Cryptosporidium** parvum, Sarcocystis muris and Toxoplasma gondii showed that the bovine Neospora isolate is genotypically unique.

DETD . . . biological samples. These sequences can be used to detect all stages of the Neospora life cycle (e.g., tachyzoites, bradyzoites, and **oocysts**) in biological samples from both the bovine host and the definitive host. A variety of methods of specific DNA and. . .

DETD . . . are particularly useful for the diagnosis of neosporosis and identification of the source of Neospora parasite stages (tachyzoites, bradyzoites and **oocysts**) in various animal hosts.

DETD . . . Neospora. The compositions of the invention can also be used to treat the definitive host to prevent the shedding of **oocysts** and subsequent transfer to cattle. The compositions for administration to either cattle or the definitive host can comprise tachyzoite and/or. . .

DETD . . . 4 different antisera to T. gondii (Tg1-4). Antiserum Tg1 was produced by the infection of a rabbit with live sporulated **oocysts** of the ME-49 strain (Lindsay & Dubey, supra) of T. gondii and used at a 1:400 dilution. Toxoplasma gondii antiserum. . .

DETD . . . antigen expression of the different parasites and variations in the methods used to produce the antisera (i.e. immunization with cysts, **oocysts** or tachyzoite lysates). For example, tissue cyst wall antigens that reacted with antiserum to H. hammondi appeared to be lacking. . .

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DETD . . . agitation. After hybridization, the membranes were washed twice

for 5 min each at room temperature in 5.times.SSC and 0.1% (w/v) **SDS**, and then washed twice for 5 min each at room temperature in 0.5.times.SSC and 0.1% (w/v) **SDS**. Membrane blocking, antibody incubations, signal generation and detection were performed as described by the manufacturer. Membranes were exposed to Kodak. . .

DETD . . . with the probes overnight at 42.degree. C. The blots were washed at 42.degree. C. (2.times.20 min.) in 6M Urea, 0.4% **SDS**, 0.5.times.SSC and rinsed with 20.times.SSC prior to autoradiography.

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DETD . . . Both recombinant antigens and parasite antigens were quantitated with the BCA Protein Assay (Pierce), denatured in Laemmli's sample buffer and **boiled** for 5 minutes prior to electrophoresis. **SDS**-PAGE and Western blots were performed under standard conditions.

L4 ANSWER 5 OF 14 USPATFULL

ACCESSION NUMBER: 2001:136774 USPATFULL

TITLE: Cloning and expression of a DNA sequence encoding a 41 kDa **cryptosporidium** parvum **oocyst** wall protein

INVENTOR(S): Jenkins, Mark C., Davidsonville, MD, United States
Fayer, Ron, Columbia, MD, United States
Trout, James, Columbia, MD, United States

PATENT ASSIGNEE(S): The United States of America as represented by the Secretary of Agriculture, Washington, DC, United States (U.S. government)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6277973	B1	20010821
APPLICATION INFO.:	US 1999-451117		19991130 (9)

DOCUMENT TYPE: Utility
FILE SEGMENT: GRANTED
PRIMARY EXAMINER: Stucker, Jeffrey
ASSISTANT EXAMINER: Winkler, Ulrike
LEGAL REPRESENTATIVE: Silverstein, M. Howard, Fado, John D., Rabin, Evelyn M.
NUMBER OF CLAIMS: 16
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 12 Drawing Figure(s); 12 Drawing Page(s)
LINE COUNT: 1510
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

TI Cloning and expression of a DNA sequence encoding a 41 kDa **cryptosporidium** parvum **oocyst** wall protein

AB . . . for the immunization of animals against cryptosporidiosis. The proteins are effective for the immunization of a variety of animals against **Cryptosporidium** parvum, particularly for the production of hyperimmune colostrum that may be used to confer passive immunity against the parasite. Isolated. . .

SUMM **Cryptosporidium** parvum is a protozoan parasite that has been implicated in numerous outbreaks of diarrheal disease in the human population. This. . . are specific for the native 41 kDa protein which specifically identifies C. parvum and thus distinguishes C. parvum from other **Cryptosporidium** species, to generate hyperimmune serum or colostrum for use in enhancing the anti-cryptosporidial response of young or immunocompromised individuals, and in vaccine development, to protect individuals from **Cryptosporidium** infection.

SUMM **Cryptosporidium** is a protozoan that can cause acute, severe, self-limited disease in immunocompetent individuals and severe chronic diarrhea in immunocompromised individuals.. . . hosts such as persons afflicted with AIDS. Development normally takes place in the intestinal epithelium and the transmissible stage, the **oocyst**, is excreted in the feces. In immunocompromised patients, cryptosporidiosis is not necessarily self-limiting and sites other than the small

intestine,. . . liver, pancreas, gall bladder, appendix, colon, rectum, and conjunctiva of the eye, may be affected (Fayer et al. 1997. In **Cryptosporidium** and Cryptosporidiosis, R. Fayer, Ed., CRC Press, New York, N.Y. page 29). Cryptosporidiosis is also a major disease of dairy and beef calves in the United States. Although a number of species of **Cryptosporidium** have been described, only C. parvum causes disease in both humans and calves.

SUMM . . . of the parasite was traced to contaminated drinking water supplied by a municipal water treatment utility. Such widespread occurrence of **Cryptosporidium oocysts** in raw and treated drinking water supplies throughout the USA has raised concern that low-level endemic waterborne **Cryptosporidium** infections may occur commonly.

SUMM **Cryptosporidium** is transmitted through animal contact, person-to-person contact, and contaminated food and water. The C. parvum infection is initiated by the ingestion of **oocysts**, the excystation of **oocysts** with release of sporozoites and the invasion of gut epithelial cells by sporozoites. Thereafter, the intracellular forms mature and release. . . sexual cycle. The sexual cycle of C. parvum also occurs in the gut and results in the production of sporulated **oocysts**, some of which may excyst before being shed. In persistent infection of an immunocompromised host, both the merozoite and the endogenously produced sporozoite may contribute to the ongoing invasion by C. parvum. **Cryptosporidium** spp. are resistant to standard disinfection processes and remain infectious for long periods of time in the environment at a wide range of temperatures. This resistance is imparted by the hard outer covering of the **oocyst** wall that surrounds the infectious stage of the parasite, ie., sporozoites.

SUMM The detection of **Cryptosporidium** parvum **oocysts** in environmental samples usually relies on one of three different techniques--vital dye staining (e.g., Modified Ziehl-Neelsen acid fast staining), direct or indirect immunofluorescence staining (IFA), or enzyme immunoassay (EIA) using **Cryptosporidium**-reactive antibodies. Differences in the relative sensitivities of these assays have been noted (Garcia et al. 1997. J. Clin. Micro. 35. . . et al. 1995. J. Clin. Microbiol. 33: 416-418). The majority of immunocompetent patients, when initially symptomatic, have large numbers of **oocysts** present in their stools and their condition can be confirmed with a number of procedures; however, as the acute infection resolves, the patient becomes asymptomatic and the number of **oocysts** dramatically decreases (Garcia et al. 1997, supra). Low numbers of **oocysts** makes identification of C. parvum as the causative agent difficult. The high sensitivity of anti-**Cryptosporidium** monoclonal antibodies (mAbs) most certainly aids detection of **Cryptosporidium** in fecal or environmental samples; however, their use does not ensure the specific detection of C. parvum, the only species that represents potential public health threats. **Cryptosporidium oocysts** shed by a variety of captive and wild homoiothermal and poikilothermal animals contaminate the surface water and water supply. In the absence of C. parvum-specific mAbs, such **oocysts** can be misinterpreted as C. parvum **oocysts** potentially pathogenic for humans based on their identification as **Cryptosporidium oocysts** by crossreactive antibodies, i.e., antibodies that react with more **Cryptosporidium** species than C. parvum (Graczyk et al. 1996. Am. J. Trop. Med. Hyg. 54(3): 274-279). Similarly, diarrhea in patients may. . . from C. parvum under circumstances where an organism other than C. parvum is the causative agent and the patient carried **Cryptosporidium oocysts** (not C. parvum) from contacts not related to the diarrheal disease, i.e., environmental contacts. This problem is of particular concern. . . of treated water destined for human consumption. None of the available immunoassays can differentiate C. parvum from other species of **Cryptosporidium** that are not infectious for mammals. The inability to sensitively detect and differentiate **Cryptosporidium** at the level of species or

subspecies (strain) is a recognized constraint on our understanding of the natural history, epidemiology, and zoonotic potential of **Cryptosporidium** isolates and therefore makes the assessment of the public health risk posed by **oocyst** contamination of water or foods difficult (M. J. Arrowood. 1997. In **Cryptosporidium** and Cryptosporidiosis, R. Fayer, Ed., CRC Press, New York, N.Y. page 56).

SUMM Confirmatory diagnosis of cryptosporidiosis in patients is often carried out by assaying sera for recognition of specific **Cryptosporidium** antigens (Frost et al. 1998. Epidemiol. Infect. 121: 205-211). Several low molecular weight C. parvum **oocyst** antigens, such as 15 kDa, 17 kDa, and 23 kDa proteins, appear to be useful for identifying the presence of **Cryptosporidium**. The immunogenicity of 15, 17, and 23 kDa antigens and somewhat higher M.sub.r antigens (e.g., 32, 47 kDa) has been observed in other mammalian species infected or immunized with C. parvum **oocysts** (Lorenzo et al. 1995. Vet. Parasitol. 60: 17-25; McDonald et al. 1992. Parasite Immunol. 14: 227-232; Nina et al. 1992.. . . 80:137-147; Reperant et al. 25 1994. Vet. Parasitol. 55: 1-13). However, laboratory studies have shown these immunodominant antigens and other **oocyst**/sporozoite proteins to be present in other **Cryptosporidium** species (Nina et al. 1992, supra; Tilley et al. 1990. Infect. Immun. 58: 2966-2971); therefore, their presence is not indicative. . . parvum infection. This cross-reactivity of immunodominant antigens may explain why commercial antibody-based tests cannot differentiate C. parvum from species of **Cryptosporidium** that are not infectious for humans.

SUMM . . . antiprotozoal drugs licensed for animal use have been approved for prophylaxis or therapy of cryptosporidiosis (Fayer et al. 1997. In **Cryptosporidium** and Cryptosporidiosis, R. Fayer, Ed., CRC Press, New York, N.Y., pages 20 and 30-31). Several researchers have shown, however, that in calves, mice and humans, administration of hyperimmune bovine colostrum, prepared by immunizing cows with extracts of C. parvum **oocysts**, can effectively confer passive immunity against cryptosporidiosis (Fayer et al. 1989. J. Parasitol. 75(1):151-153; Fayer et al. 1989. J. Parasitol.. . . colostrum have been reported to ameliorate C. parvum infection in AIDS or other immunocompromised patients. Hyperimmune bovine colostrum prepared against **oocysts** contains neutralizing antibodies that recognize epitopes expressed by all life-cycle stages of **Cryptosporidium**.

SUMM . . . and either prevent or lessen the severity of infection in animals. The characteristics of many mAbs which specifically react with **Cryptosporidium** have recently been reviewed; many are neutralizing (Riggs. 1997. In **Cryptosporidium** and Cryptosporidiosis, R. Fayer, Ed., CRC Press, New York, N.Y., Chapter 6). In some instances, the epitope recognized by the. . .

SUMM . . . recombinant antigens can be used to prepare monoclonal antibodies (mAb) which selectively identify or which are specific for C. parvum **oocysts**. These mAbs can be used in ELISA and in IFA to detect the parasite in diarrhea or in water samples similar to **Cryptosporidium** spp.-binding mAb used in commercial diagnostic kits (e.g., Merifluor). Third, the recombinant antigens can be used to generate hyperimmune serum. . . particularly RT-PCR. PCR assays have been developed in a number of laboratories, including our own, to detect less than 10 **oocysts** in a spiked water sample, but the previously identified primers, as well as primers generated from the nucleotide sequences of SEQ ID NO:1, amplify DNA from all species of **Cryptosporidium**. However, primers can be used in RT-PCR to specifically identify transcription of C. parvum-specific proteins.

SUMM We have now discovered a novel recombinant DNA clone designated rCP41 which encodes an **oocyst** wall protein of **Cryptosporidium** parvum, which may have immunodiagnostic potential for cryptosporidiosis as well as potential for use in the production of hyperimmune colostrum. . .

SUMM . . . an immune response specific for C. parvum. This invention, in addition to the above, also encompasses a method of diagnosing **Cryptosporidium** infection of a subject, comprising: contacting a

body fluid obtained from the subject with the peptide of this invention; and detecting any selective binding of the peptide to any anti-**Cryptosporidium** antibodies in the body fluid.

SUMM In particular, this invention comprises a method of diagnosing **Cryptosporidium** infection of a subject, comprising: contacting a body substance obtained from the subject with an anti-C. parvum antibody; and detecting. . .

SUMM Further, as a public health issue, there is a need for a method to identify and enumerate the presence of **Cryptosporidium** parvum in water. This invention comprises a method of identifying the presence of C. parvum in water, comprising: contacting a. . .

SUMM Another object of the invention relates to a method of inhibiting or ameliorating a **Cryptosporidium** infection in an individual comprising administering to an individual in need of such treatment an amount of an anti-CP41 or. . .

SUMM . . . sequence shown in SEQ ID NO:2 and wherein said protein is antigenic and effective to elicit an immune response against **Cryptosporidium** parvum in a host animal and a second unrelated peptide expressed by a regulatory DNA segment operably coupled to the. . . sequence shown in SEQ ID NO:2 and wherein said protein is antigenic and effective to elicit an immune response against **Cryptosporidium** parvum in a host animal operably coupled to yet another unrelated polypeptide sequence (different from the regulatory protein). It is. . .

SUMM Also part of this invention is a **Cryptosporidium** diagnostic kit, comprising anti-**Cryptosporidium**-specific antibodies; and instructions for the use of the kit.

SUMM Furthermore, this invention also provides a **Cryptosporidium** diagnostic kit, comprising the proteins and peptides of this invention; and instructions for use of the kit.

DRWD FIG. 1 shows the immunostaining of **SDS**-PAGE fractionated native **Cryptosporidium** parvum (Cp) or C. baileyi (Cb) **oocyst** protein, or NiNTA-purified (P) and unpurified (IP) recombinant CP41 protein (rCp41) with rabbit sera to whole C. parvum **oocyst** protein (R .alpha. CpOO), native (R .alpha. NATIVE Cp41) or recombinant (R .alpha. RECOMBINANT Cp41) Cp41 antigen, or normal control. . .

DRWD FIG. 2 shows the DNA sequence and the predicted amino acid sequence of CP41 DNA clone isolated from **Cryptosporidium** parvum **oocysts**. Putative ATG start sites are indicated in bold.

DRWD FIG. 3 shows PCR amplification of the CP41 sequence from genomic DNA (equivalent to 10.sup.3 **oocysts**) from a bovine isolate of **Cryptosporidium** parvum (Cp-bov), a human isolate of C. parvum (Cp-hu), C. baileyi (Cb), or C. wrairi (Cw). Kbp, .phi.x174 DNA standards.

DRWD FIG. 4 shows the molecular analysis of **Cryptosporidium** parvum for presence of CP41 sequence in genomic DNA using PCR, and for CP41 mRNA in total RNA using RT-PCR. The PCR assay was performed on DNA equivalent to 10.sup.3 C. parvum **oocysts** (CP DNA). The RT-PCR was performed on total RNA equivalent to 5.times.10.sup.5 C. parvum **oocysts** stored at 4.degree. C. for 6 mo. (CP1), 3 mo. (CP2), or 1 mo. (CP3). +, Superscript reverse transcriptase (Rtase). . .

DRWD FIG. 5 shows the immunofluorescence staining of **Cryptosporidium** parvum **oocysts** with rabbit antisera prepared against native CP41 protein (A), or recombinant CP41 antigen (B), or with normal control rabbit sera. . .

DRWD FIG. 6 shows immunoelectron microscopy staining of **Cryptosporidium** parvum **oocysts** with rabbit antisera prepared against native CP41 protein (A), or recombinant CP41 antigen (B). Bar=100 nm.

DRWD FIG. 7 shows serological titers against recombinant CP41 (.box-solid.-.box-solid.) and native **Cryptosporidium** parvum **oocyst** protein (.tangle-solidup.-.tangle-solidup.) in four calves (A-D) exposed to a natural C. parvum infection and one calf (E) exposed to an experimental natural C. parvum infection as revealed by ELISA. Arrow indicates first day of C. parvum **oocyst** shedding.

DETD . . . 41 kDa protein, CP41, and rCP41, the recombinant 36 kDa and 28 kDa proteins, all of which are specific for **Cryptosporidium** parvum and the nucleic acid sequences that encode these proteins. Antibodies resulting from immunizations with the recombinant 36 and 28. . .

DETD . . . species-specific PCR test based on genomic DNA, but that the CP41 primers are useful for species-specific RT-PCR analysis of total **Cryptosporidium** RNA (Example 14).

DETD . . . proteins of this invention in monomeric or multimeric form can be incorporated into vaccines capable of inducing protective immunity against **oocysts**/sporozoites of C. parvum. The peptides or proteins of this invention can be administered as multivalent subunit vaccines in combination with other antigens of C. parvum. For example, they may be administered in conjunction with other **oocyst** /sporozoite components of C. parvum. Furthermore, it will be understood that peptides specific for a plurality of C. parvum stages and **Cryptosporidium** species may be incorporated in the same vaccine composition to provide a multivalent vaccine. In addition, the vaccine composition may. . .

DETD . . . al., referred to above. In addition, the method used by Jenkins et al. to successfully obtain high levels of colostral anti-**Cryptosporidium** antibodies is described in U.S. Pat. No. 5,591,434, the contents of which are herein incorporated by reference. Briefly, purified recombinant. . .

DETD Also an important part of this invention is a method of diagnosing **Cryptosporidium** infection, that comprises contacting a body substance with an anti-**Cryptosporidium** antibody having specificity for the polypeptide of this invention; and detecting any selective binding of the antibody to any antigenic **Cryptosporidium** peptides present in the body substance. The anti-**Cryptosporidium** antibodies may be monoclonal or polyclonal. Also provided herein is a method of detecting the presence of **Cryptosporidium** parvum in water samples. The detection of the antibody-polypeptide complex may be conducted by any method known in the art.. . .

DETD . . . and fluorescence signal on a flow cytometer. Positive control seeded samples have shown a linear correlation with the number of **oocysts** recovered from the gradients (Arrowood et al. 1995, supra). Flow cytometric analysis of stool samples from infected bovine and human. . .

DETD . . . or concentrate the low numbers of C. parvum in the sample. For example, C. parvum can be concentrated using biotin-labeled anti-**Cryptosporidium**-specific mAbs, together with anti-biotin-labeled magnetic beads. Because of the microscopic size of the MAC beads, any **oocysts** positively selected by this method can then be stained with FITC-anti-rCP41 mAb, specific for C. parvum, and rapidly and specifically. . .

DETD In a most preferred embodiment of the presently claimed diagnostic methods for identifying presence of C. parvum **oocysts**, the method comprises the steps of: collecting a water sample; isolating an **oocyst**-rich fraction from the water sample by concentration with immunomagnetic methodology; staining the concentrated **oocysts** with C. parvum-specific mAb, and determining the actual number, if any, of C. parvum **oocysts**.

DETD Additionally provided herein is a method of diagnosing **Cryptosporidium** infection, that comprises contacting a body substance with one of the polypeptides of this invention; and detecting any selective binding of the polypeptide to any anti-**Cryptosporidium** antibodies in the body substance. As in the previous case, the present antibody-polypeptide binding complex may be detected by a. . .

DETD Still part of this invention is a kit for the diagnosis of **Cryptosporidium** infection, that comprises the peptide(s) of this invention; and instructions for use of the kit. This kit may be utilized. . . with cryptosporidiosis. Even at the early stages where the parasite is commencing invasion of a subject's cells, some amount of

Cryptosporidium specific antibody may be detected in serum.

DETD Also provided herein is another **Cryptosporidium** diagnostic kit, that comprises anti-**Cryptosporidium** antibodies having specificity for one of the polypeptides of this invention; and instructions for use of the kit. Thus, kit may be utilized for the detection of **Cryptosporidium** peptides, a sign that there is parasite present in the subject being tested.

DETD **Cryptosporidium** parvum (AUCP-1) strain **oocysts** were obtained by infecting a 1 day old calf with 10.sup.6 **oocysts**. The calf was obtained at birth from the dairy herd at the Beltsville

DETD . . . the procedures described by Kilani et al. 1987. Am. J. Trop. Med. Hyg. 36: 505-508) for purification of C. parvum **oocysts**. Clean **oocysts** were resuspended in distilled water, stored at 4.degree. C., and used from 1-6 months after collection, depending on objectives of the experiment. Limited numbers of **oocysts** of other **Cryptosporidium** species were obtained from outside sources: C. baileyi (B. Blagburn, Auburn University), C. meleagtidis (M. Levy, North Carolina State University),. . .

DETD **Cryptosporidium oocysts** destined for RNA and DNA extraction were treated for 30 min. with 2.5% sodium hypochlorite (50% Clorox), washed 5 times. . . step was incorporated as per instructions from the manufacturer to remove polysaccharide which appears to exist in large quantities in **Cryptosporidium**. DNA of C. parvum was prepared by treating the parasite extract with 1% sodium dodecylsulfate (**SDS**) and 50 .mu.g/ml proteinase K (Gibco/BRL, Gaithersburg, Md.) as described (Jenkins et al. 1993. Infect Immun. 61: 2377-2382). RNA and DNA yields were estimated by O.D..sub.260 /O.D..sub.280 reading. Total **oocyst** protein was prepared by resuspending the parasites in protein extraction buffer (10 mM Tris-HCl pH 7.3, 1 mM MgCl.sub.2) in the presence of phenylmethylsulfonyl-fluoride (PMSF). The **oocysts** were subjected to five freeze-thaw cycles between dry ice-EtOH and 37.degree. C. water baths.

DETD **SDS**-PAGE/immunoblotting of Native and Recombinant C. parvum Protein

DETD Protein extracts of **Cryptosporidium oocysts** were treated with sample buffer containing 2-mercaptoethanol, heated for 3 min. in a **boiling** water bath, fractionated by 7.5-15% gradient **SDS**-PAGE, and transblotted to Immobilon (Millipore, Bedford, Mass.) membrane as described (Jenkins et al. 1993, supra). The antigen-impregnated membranes were treated. . .

DETD Identification of **Cryptosporidium** parvum-specific 41 kDa Protein

DETD Total **oocyst** protein extracted from C. parvum, C. baileyi, C. meleagridis, and C. serpentis was electrophoresed in adjacent lanes of a 7.5%-15% gradient **SDS**-PAGE (10.sup.7 **oocysts**/lane) and transblotted to nitrocellulose membrane (Schleicher and Schuell, Keene, N.H.). The blots were immunostained with rabbit antisera raised against total C. parvum **oocyst** protein to identify antigens unique to C. parvum.

DETD Antisera raised against total C. parvum **oocyst** protein recognized a number of antigens of C. parvum and C. baileyi (FIG. 1). A similar recognition pattern was observed with C. meleagridis and C. serpentis (data not shown). Although the majority of antigens were common to all **Cryptosporidium** species, a few antigens were unique to C. parvum. In particular, a 41 kDa protein was present in C. parvum but not in the other **Cryptosporidium** species (FIG. 1).

DETD Preparation of Antisera to **Cryptosporidium** parvum 41 kDa Protein

DETD Immunoscreening **Cryptosporidium** parvum Genomic DNA Libraries for p41 Clones

DETD The **Cryptosporidium** insert DNA was excised from pBluescript by PstI and HindIII digestion and ligated into the pTrcHis A vector (Invitrogen, Carlsbad,. . .

DETD Immunoblots of **SDS**-PAGE fractionated protein extracts from E. coli harboring recombinant pTrcHis-CP41 plasmid contained two unique protein bands, 28 and 36 kDa, produced. . .

DETD . . . shown). Antisera raised against native or recombinant CP41 antigen showed a similar pattern in immunoblots with whole native C. parvum **oocyst** protein (Cp), purified (P) or unpurified (IP) recombinant CP41 protein (FIG. 1). Normal control serum showed negligible recognition of native. . .

DETD . . . proteins did not appear to be due to glycan residues on the native protein. Glycosidase treatment was performed on total **oocyst** extracts to determine if CP41 was glycosylated. In addition, untreated **SDS**-PAGE-fractionated C. parvum protein impregnated on Immobilon membrane was treated with sodium perodiate to remove glycan residues. No difference in molecular. . .

DETD . . . and 28 kDa recombinant proteins may be due to the previously reported aberrant migration of E. coli-expressed recombinant proteins in **SDS**-PAGE for proteins with atypical amino acid compositions. The CP41 coding sequence contains a disproportionate number of Asn (21%) and Thr. . .

DETD C. parvum sporozoites were first excysted by treating **oocysts** with 1% sodium hypochlorite (20% Clorox) for 10 min. at room temperature (RT), washing 5 times with Hank's balanced salt. . . for 1 hr in a humidified chamber at 37.degree. C. in 5% CO.sub.2. For examination by immunofluorescence microscopy, the excysted **oocyst**/sporozoite mixture was pipetted onto multi-well glass slides at 10.sup.4 **oocysts** per well (based on pre-excystation counts) and allowed to air dry at room temperature (RT). The parasites were then treated. . .

DETD The C. parvum **oocysts**/sporozoites were examined on an epifluorescence microscope. Antisera to native or recombinant CP41 antigen bound a surface antigen of C. parvum **oocysts** (FIG. 5). Although not evident from the IFA figures, the target antigen appeared to be distributed unevenly on the surface of the **oocyst**. Negligible staining of C. baileyi **oocysts** was observed with antisera to native or recombinant CP41 antigen (data not shown).

DETD For IEM, the excysted sporozoite/**oocyst** mixture was washed several times in PBS, enumerated on a hemocytometer, aliquoted at 10.sup.8 sporozoites into 1.5 ml microcentrifuge tubes, and pelleted by centrifugation. The **oocyst**/sporozoite pellet was resuspended in 2% paraformaldehyde, 0.5% glutaraldehyde in 0.1M sodium cacodylate for 20 min. at RT. The fixed parasites were washed twice with 0.1M sodium cacodylate and pelleted by centrifugation. The **oocyst** /sporozoite pellet was dehydrated in a graded ethanol series, infiltrated overnight in LR White hard grade acrylic resin, and cured at. . .

DETD . . . by IFA was confirmed by IEM (FIG. 6). The antigen was present on both external and internal regions of the **oocyst** wall and was also associated with amorphorous material on the **oocyst** outer surface.

DETD Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) Analysis of **Cryptosporidium** parvum **oocyst** RNA

DETD C. parvum **oocysts** stored for 1, 3, or 6 mos. at 4.degree. C. were analyzed for the presence of CP41 messenger RNA using. . . To analyze for the presence of CP41 in genomic DNA, a single PCR was performed on DNA equivalent to 10.sup.3 **Cryptosporidium** **oocysts** using reaction conditions identical to those described above. The PCR products were analyzed by polyacrylamide gel electrophoresis, EtBr staining and. . .

DETD RT-PCR on total RNA showed that the CP41 transcript was present in C. parvum **oocysts** (FIG. 4). Although equivalent amounts of total RNA were used in each reaction, the intensity of the RT-PCR signal appeared to be inversely correlated with the age of C. parvum **oocysts**. The RT-PCR signal derived from RNA isolated from **oocysts** that had been stored at 4.degree. C. for 6 mo (CP1+) or 3 mo (CP2+) was about 25% and 50% respectively, of the signal obtained from 1 mo old **oocysts** (CP3+, FIG. 3). The control reactions in which reverse transcriptase was not included did not show a PCR product indicating. . .

DETD . . . had experience sporadic outbreaks of cryptosporidiosis (natural

infection). The calves were examined daily for diarrhea and excretion of C. parvum **oocysts** in feces using standard methods. Antisera was also obtained from a calf prior to and every week for one month after being fed NBC at birth and inoculated per os with 10.sup.6 C. parvum **oocysts** (experimental infection). Antisera were also obtained from adult cows (3-4 years old) before and for several weeks after experimental C.. . . cows that had not been exposed to C. parvum. Antisera were tested by ELISA for recognition of native C. parvum **oocyst** protein and recombinant CP41 antigen. In the former, C. parvum **oocysts** were subjected to multiple freeze-thaw cycles and disruption on a Mini-bead beater (Bespeak Products, Bartlesville, Okla.) followed by centrifugation to pellet insoluble material. The **oocyst** protein supernatant (50 .mu.l), equivalent to 4.times.10.sup.4 **oocysts** (150 ng protein), was pipetted onto individual wells of Immulon II microtiter plates and incubated overnight at 4.degree. C. Native. . . by denaturing NiNTA affinity chromatography according to manufacturer's directions (Invitrogen). Eluates containing peak amounts of purified protein as indicated by **SDS**-PAGE/immunoblotting were pooled and adsorbed to the surface of Immulon II microtiter plates as described above. The wells were washed with. . .

DETD Recombinant CP41 antigen showed levels of binding similar to native C. parvum **oocyst** antigen when probed with sera from adult cows that were exposed to C. parvum (Table 1). The pre-infection titers to. . . and did not appear to increase appreciably after experimental challenge. Perhaps boosting of the antibody response was not observed because **oocyst** challenge did not result in patent infection due to the age-related resistance of adult cows to C. parvum infection. In. . . increase in Ab titers to rCP41 was noted after exposure to C. parvum. The Ab titers to native C. parvum **oocyst** antigen were variable. In two calves (FIGS. 7B, 7D), the highest titers against native antigen occurred at time of **oocyst** shedding. In the other two calves, peak anti-C. parvum antigen titers were observed immediately after colostrum feeding (FIGS. 7A, 7C).. . . after an experimental C. parvum infection of a one-day-old calf (FIG. 7E). Antibody titers to rCP41 and native C. parvum **oocyst** antigen were highest after colostrum feeding and natural C. parvum **oocyst** inoculation (FIG. 7E). Although anti-rCP41 titers decreased with time, the response to native C. parvum **oocyst** titers was variable (FIG. 7E).

DETD TABLE 1

Anti-recombinant Cp41 and native **Cryptosporidium** parvum **oocyst** antigen titers in sera from adult cows after experimental cryptosporidiosis infection or in unexposed control adult cows.

Cow No.	Time Post-infection (wk)	ELISA Titer rCP41	nCP **oocyst**
Experimental Infections:			
1	0	1100	2560
	1	1310	ND*
	2	1260	1280
	3	1200	2560
	4	1110	2560
2	0	900	2560
	1	1020.	. .

DETD . . . hrs after the fusion. Hybridoma supernatants were screened by ELISA using rCP41 antigen and by immunofluorescence assay using C. parvum **oocysts** dried to the surface of multi-well glass slides. Cloning by limiting dilution was performed until a single clone was observed. . .

CLM What is claimed is:

. . . sequence shown in SEQ ID NO:2 and wherein said protein is antigenic and effective to elicit an immune response against **Cryptosporidium** parvum in a host animal.

. . . an amino acid sequence shown in SEQ ID NO:2 and is antigenic and effective to elicit an immune response against **Cryptosporidium** parvum in a host animal.

. . . sequence shown in SEQ ID NO:2 and wherein said protein is antigenic and effective to elicit an immune response against **Cryptosporidium** parvum in a host animal.

. . . sequence shown in SEQ ID NO:2 and wherein said protein is antigenic and effective to elicit an immune response against **Cryptosporidium** parvum in a host animal, operably coupled to another unrelated polypeptide sequence.

11. The vector of claim 10, wherein the unrelated polypeptide sequence is another **Cryptosporidium** parvum antigenic peptide or a **Cryptosporidium** stage-specific peptide.

L4 ANSWER 6 OF 14 USPATFULL

ACCESSION NUMBER:	2000:153489 USPATFULL		
TITLE:	Method for the detection of viable **Cryptosporidium** parvum **oocysts**		
INVENTOR(S):	Williams, Keith Leslie, Frenchs Forest, Australia Vesey, Graham, Drummoyne, Australia Veal, Duncan, Turramurra, Australia Ashbolt, Nicholas John, Potts Point, Australia Dorsch, Matthias, Lane Cove, Australia		
PATENT ASSIGNEE(S):	Macquarie Research, Ltd., Sydney, Australia (non-U.S. corporation) Australian Water Technologies Pty. Ltd., Sydney, Australia (non-U.S. corporation)		
	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6146855		20001114
	WO 9634978		19961107
APPLICATION INFO.:	US 1998-952376		19980303 (8)
	WO 1996-AU274		19960506
			19980303 PCT 371 date
			19980303 PCT 102(e) date
	NUMBER		DATE
PRIORITY INFORMATION:	AU 1995-2831		19950505
DOCUMENT TYPE:	Utility		
FILE SEGMENT:	Granted		
PRIMARY EXAMINER:	Arthur, Lisa B.		
ASSISTANT EXAMINER:	Enewold, Jeanine		
LEGAL REPRESENTATIVE:	Barnes & Thornburg, Martin, Alice O.		
NUMBER OF CLAIMS:	11		
EXEMPLARY CLAIM:	1,4		
NUMBER OF DRAWINGS:	3 Drawing Figure(s); 4 Drawing Page(s)		
LINE COUNT:	516		

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

TI Method for the detection of viable **Cryptosporidium** parvum **oocysts**

AB Oligonucleotide molecules and methods are disclosed for the detection of viable **oocysts** or other cells of the protozoa species, Cyrptosporidium parvum. Preferred oligonucleotide molecules are selected from the group comprising oligonucleotides having. . .

SUMM The present invention relates to a method for the detection of viable **oocysts** or other cells of the protozoa species Cryptosporodium parvum. More particularly the invention relates to a method for such detection. . .

SUMM The detection of **Cryptosporidium oocysts** in water presently relies on the concentration of particulate matter from large

volumes of water prior to staining with fluorescently labelled monoclonal antibodies. Until recently, detection and identification of fluorescently labelled **oocysts** required examination of the sample using epifluorescence microscopy. The tedious and labour intensive nature of this detection method,. in particular. . . assisted detection methods has alleviated some of these problems and enabled the routine monitoring of water for the presence of **Cryptosporidium oocysts** (Vesey et al., 1994A). However, a major limitation of all these methodologies is the lack of **oocyst** viability measurements. Methods to determine viability such as animal infectivity and excystation, are impractical because of the low number of **oocysts** normally present in water samples and the tedious nature of such tests.

SUMM The presence of dead **Cryptosporidium oocysts** in drinking water is of little significance to public health, however if **oocysts** are viable the risk to public health is enormous. Moreover, of the seven species of **Cryptosporidium**; C. parvum, C. muris, C. meleagridis, C. serpentis, C. nasorum, C. wrairi and C. baileyi only one, C. parvum is. . . species specific. There is therefore, an urgent requirement to develop an effective method for determining the viability of C. parvum **oocysts** in water.

SUMM In a first aspect, the present invention provides an oligonucleotide molecule for the detection of viable cells of **Cryptosporidium** parvum (C. parvum), the oligonucleotide molecule characterised in that it specifically hybridises to unique 18S rRNA sequences of C. parvum.

SUMM . . . be used to detect the presence in a sample of any type of viable cell of C. parvum. Normally only **oocysts** will be found in environmental samples. Other cell types may, however, be found and detected in clinical samples.

DRWD FIG. 1 shows the results of flow cytometric analysis of FISH stained **oocysts** using a eukaryotic specific probe (Euk probe) and a bacterial specific probe (Bac probe).

DRWD FIG. 2 shows the comparison of **oocyst** viability as measured by excystation and viability determined by FISH on **oocysts** following storage at 22.degree. C. in the dark and sampled over a 74 day period.

DETD **Cryptosporidium** parvum. **Cryptosporidium** parvum **oocysts** cultured in lambs and purified by density gradient centrifugation were purchased from the Moredun Animal Research Institute, Edinburgh. UK.

DETD . . . (Euk) complementary to a 18S rRNA region conserved for Eucarya (5'-ACCAGACTTGCCCTCC-3') (Amann et al., 1990) was used to stain Cryptosporiduium **oocysts**. A second probe (Bac) complementary to a 16S rRNA region conserved for all bacteria (5'-GCTGCCTCCCGTAGGAGT-3') (Amann et al., 1990) was. . .

DETD Extraction of genomic DNA: **Oocysts** were pelleted by centrifugation, resuspended in TE buffer, and repeatedly frozen in a mixture of dry ice and ethanol and thawed by **boiling** for 2 mins. After incubation with **SDS** and proteinase K (1% w/v 100 .mu.g/ml respectively)for 1 h at 37.degree. C. the lysate was extracted with phenol, phenol-chloroform. . .

DETD TABLE 1. Sequences for preparing specific probes for target sites of 18S ribosome for **Cryptosporidium** parvum. The position of the target sites with respect to the whole 18S rRNA sequence are shown in FIG. 3.

DETD Fixation of **oocysts**. Fixation of **oocysts** was performed using a modified method of that described previously by Wallner et al. (1993) for the fixation of yeasts and bacteria. One volume of **oocyst** suspension containing approximately 10.sup.7 **oocysts**, was mixed with three volumes of fresh cold 4% w/v paraformaldehyde in phosphate buffered saline (PBS), pH 7.2, and kept at 4.degree. C. for 1 h. The **oocysts** were washed three times by centrifugation (13.000 g. 30 s) and then resuspended in PBS. The sample was then mixed. . .

DETD Hybridisation. Fixed **oocysts** were hybridised with the probe by mixing 10 .mu.l of **oocyst** suspension with 100 .mu.l of

hybridisation buffer (0.9 M NaCl, 20 mM Tris/HCl pH 7.2, 0.5% w/v sodium dodecylsulfate) prewarmed. . .

DETD . . . Nikon Optiphot-2 microscope fitted with differential interference contrast (DIC) optics and excitation and emission filters suitable for the examination FITC. **Oocysts** were detected using DIC and then examined for fluorescence. A minimum of 100 **oocysts** were examined in each sample.

DETD Excystation. In vitro excystation was performed as described by Campbell et al. (1992). To a 100 .mu.l volume of **oocyst** suspension (approximately 10.sup.4 **oocysts**), 10 .mu.l of 1 (w/v) sodium deoxycholate in Hanks minimal essential medium and 10 .mu.l of 2.2% sodium hydrogen carbonate. . . added. After incubation, .sub.37 .degree. C. for 4 h, samples were examined microscopically using DIC optics. The proportion of empty **oocysts**, partially excysted **oocysts** and non-excysted **oocysts** were determined. At least 100 **oocysts** were counted in each sample. The percent excystation was calculated as follows:

DETD (number of empty **oocysts**+number of partially excysted **oocysts**)/.times.100 total number of **oocysts** counted

DETD where the number of empty **oocysts** equalled the number pre-excystation subtracted from the number post excystation.

DETD . . . determine if samples could be fixed and then stored before analysis, storage experiments were performed. Aliquots (100.mu.l) of fixed **oocyst** suspensions were stored at 4.degree. C. for 1 month. Samples were removed at time intervals, stained with FISH using the. . .

DETD Aging of **oocysts**. Aliquots (10 .mu.l) of **oocyst** suspensions containing 10.sup.8 **oocysts** were diluted in 10 ml of PBS and stored at 22.degree. C. in the dark. Samples (0.5 ml) were taken at time intervals and the viability of **oocysts** assessed using both FISH with the eukaryotic specific probe and excystation. All experiments were performed in triplicate.

DETD Staining of **oocysts** with FISH. Microscopic examination of **oocysts**, which had been stained using fluorescence in situ hybridisation with the Euk rRNA probe, revealed brightly fluorescent **oocysts** together with **oocysts** which showed no fluorescence. Fluorescent staining was located within the sporozoites. Examination of the fluorescent **oocysts** using DIC optics revealed intact **oocysts** with a small gap between the **oocyst** wall and the internal structures. In comparison, non-fluorescent **oocysts** frequently appeared to have a ruptured **oocyst** wall and a large gap between the **oocyst** wall and the internal structures. Empty **oocysts** did not fluoresce.

DETD Flow cytometric analysis of **oocysts** stained by FISH with the Euk rRNA probe resulted in two distinct populations, a brightly fluorescent population and a non-fluorescent. . . epifluorescence microscopy and flow cytometry of samples which had been stained with the Bac probe resulted-in no fluorescence in any **oocysts** above that of the autofluorescence of unstained occysts (FIG. 1).

DETD Stored samples. Samples of fixed **oocysts** which had been stored at 4.degree. C. for up to 4 weeks and then stained with FISH using the Euk probe showed no reduction in the number of **oocysts** which fluoresced and no reduction in the brightness of fluorescence (Table 2).

DETD TABLE 2. A comparison of the fluorescence intensity of freshly fixed **oocysts** and **oocysts** fixed and stored at 4.degree. C. for up to 3 weeks, before staining with FISH and analysed using flow cytometry.

DETD Comparison of viability determined by FISH and in vitro excystation. A comparison of **oocyst** viability, measured by excystation and staining by FISH with the Euk and CRY1 probes on batches of **oocysts** obtained from Moredun Animal Research Institute are presented in Table 3. Results were very similar for both methods of assessing **oocyst** viability indicating that the rRNA probes only stain viable **oocysts**.

DETD TABLE 3. Comparison of **oocyst** percentage viability determined by excystation and viability determined by FISH on three different

batches of **oocysts**..sup.1
DETD . . . 56% (1.2).sup.(2)
76% (3.7) 95% (2 2)
71%
Excystation 54% (2 7) 79% (2.8) 92% (4 9) 69%

.sup.1 100 **oocysts** were examined for each determination.
.sup.2 Standard Deviation.

DETD .sup.1 100 **oocysts** were examined for each determination.

DETD Comparison of FISH and excystation for determining **oocyst** viability on suspensions of **oocysts** stored at 22.degree. C. in the dark are presented in FIG. 2. Results are very similar for both methods. A gradual decline in the viability of the **oocysts** from 90% to 40% over the 74 day period was observed with both methods. Correlation of the two sets of. . .

DETD The present inventors have developed a method using FISH and a rRNA directed probe to assess the viability of **Cryptosporidium** sp. **oocysts**. However, the invention is not restricted to **oocysts**. The unique 18S rRNA sequence of C. parvum identified and the method of the detection as stated, may also be. . .

DETD **Oocysts** containing fluorescing sporozoites after hybridisation with the probes are viable and **oocysts** which do not fluoresce are dead. The reason that dead **oocysts** do not stain is because the rRNA which the probes bind to deteriorates rapidly and in dead **oocysts** is not present in sufficient copy numbers to be detected.

DETD In vitro excystation is currently considered the gold standard to which methods for determining **oocyst** viability are compared. Results from comparing measurement of **oocyst** viability using FISH and measuring viability using in vitro excystation produced very similar results with both methods for all samples of **oocysts** analysed. Correlation of the FISH assay with excystation was highly statistically significant with a calculated correlation coefficient of 0.998. Furthermore, the FISH method was found to be easy to perform and the results easily interpreted. **Oocysts** were either fluorescent, indicating a viable **oocyst**, or did not fluoresce at all, indicating a dead **oocyst**.

DETD . . . be analysed immediately as storage or fixation of the samples will result in a reduction in the viability of the **oocysts** as measured by this technique.

DETD Campbell, A. T., Robertson, L. J., Smith, H. V. (1992). Viability of **Cryptosporidium** parvum **oocysts**: correlation of in vitro excystation with inclusion of fluorogenic vital dyes. Appl. Environ. Microbiol., 58: 3488-3493.

DETD Campbell, A. T., Robertson, L. J., Smith, H. V. (1993A). Effects of preservatives on viability of **Cryptosporidium** parvum **oocysts**. Appl. Environ. Microbiol., 59: 4361-4362.

DETD Campbell, A. T., Robertson, L. J., Smith, H. V. (1993B). Novel methodology for the detection of **Cryptosporidium** parvum: A comparison of cooled charge coupled devices (CCD) and flow cytometry. Wat. Sci. Tech., 27: 89-92.

DETD Robertson, L. J., Campbell, A. T., Smith. H. V. (1992). Survival of **Cryptosporidium** parvum **oocysts** under various environmental pressures. Appl. Environ. Microbiol., 58: 3494-3500.

DETD Vesey, G., Slade, J. S., Bvrne, M., Shepherd, K., Dennis, P. J. and Fricker, C. R. (1993). Routine monitoring of **Cryptosporidium** **oocysts** in water using flow cytometry. J. Appl. Bacteriol., 75: 87-90.

CLM What is claimed is:

1. An oligonucleotide molecule for the detection of viable **oocysts** of **Cryptosporidium** parvum (C. parvum) in an environmental sample to be tested for safety of human use, wherein said molecule specifically hybridizes. . . (SEQ ID NO: 5); and (f) CTT TTT GGA (SFQ ID NO: 6); wherein the molecule allows detection of viable **oocysts** of C. parvum.

. . . molecule of claim 1, wherein the molecule hybridizes under conditions of moderate or high stringency to rRNA of C. parvum **oocysts**.

4. A method for detection of viable **oocysts** of **Cryptosporidium** parvum (C. parvum) present in an environmental sample to be tested for safety of human use, the method comprising the. . . adding to the sample an oligonucleotide molecule of claim 1 which is detectably labeled; (b) permeabilising or lysing C. parvum **oocysts** present in the sample; (c) allowing hybridization between the oligonucleotide molecule and the 18S rRNA of the permeabilised or lysed C. parvum **oocysts**; and (d) detecting the hybridization of the molecule to the rRNA in the sample from which detection of the viable **oocysts** is inferred.

10. A method for tie detection of the presence of viable **oocysts** of **Cryptosporidium** parvum (C. parvum) in an environmental sample, the method comprising: (a) adding to the sample a first and a second. . . to claim 1, and the second primer hybridizes to 18S rRNA of C. parvum; (b) permeabilising or lysing C. parvum **oocysts** present in the sample to allow hybridization of the primer to the 18S rRNA of any C. parvum **oocysts** present in the sample; (c) conducting a polymerase chain reaction (PCR) to amplify a part of the 18S rRNA or. . . and (d) detecting the amplified part of the rRNA or rDNA in the sample from which the presence of viable **oocysts** of C. parvum is inferred.

L4 ANSWER 7 OF 14 USPATFULL
ACCESSION NUMBER: 2000:113702 USPATFULL
TITLE: Apparatus and method for nucleic acid isolation using supercritical fluids
INVENTOR(S): Nivens, David E., Knoxville, TN, United States
Applegate, Bruce M., Knoxville, TN, United States
PATENT ASSIGNEE(S): National Water Research Institute, Fountain Valley, CA, United States (U.S. corporation)
Critical Point Technologies, Inc., Knoxville, TN, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6110674		20000829
APPLICATION INFO.:	US 1997-892540		19970714 (8)
RELATED APPLN. INFO.:	Division of Ser. No. US 1996-733816, filed on 18 Oct 1996, now patented, Pat. No. US 5922536		

DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Housel, James C.
ASSISTANT EXAMINER: Ryan, V.
LEGAL REPRESENTATIVE: Knobbe, Martens, Olson & Bear, LLP
NUMBER OF CLAIMS: 7
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 4 Drawing Figure(s); 4 Drawing Page(s)
LINE COUNT: 813
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
SUMM . . . or combinations of these, followed by dissolution of the cell membrane with alkali and detergents such as sodium dodecyl sulfate (**SDS**) (Maniatis et al., 1989; Tsai et al., Appl. Environ. Microbiol., 57:1070-1074, 1991; Bej et al., Appl. Environ, Microbiol., 57:1013-1017, 1991).. . .
DETD . . . lysate rather than removing the lysate from the lytic agent. The supercritical properties of CO.sub.2 thus eliminate the need for **SDS** as a lytic reagent. These current art procedures not only result in loss of sample, but in some cases are. . .
DETD . . . methods allowing a more rapid detection of microorganisms contained in water samples, including waterborne pathogens such as E. coli, Shigella, **Cryptosporidium** and Giardia. The apparatus can

be used to detect microorganisms present in recreational waters, source water and potable water.

DETD . . . Mycobacterium and Rhodococcus. Results from lysis of these species under supercritical conditions were compared to those obtained using a conventional SDS lysis procedure (Table 1). For Pseudomonas, Sphingomonas and E. coli, the lytic action of supercritical fluids is comparable to that of **SDS** and, in the case of Rhodococcus and Mycobacterium, supercritical fluids are a better lytic agent. Dilution plate counts of lysed bacteria showed no growth after treatment with **SDS** or exposure to supercritical conditions, indicating that both methods result in loss of bacterial viability.

DETD TABLE 1

	% SFE Lysis by AODC count	Standard Deviation	% **SDS** Lysis by AODC count	Standard Deviation
E. coli	88.83	2.98	90.23	1.85
Pseudomonas	90.31	6.88	94.09	8.39
Sphingomonas				

. . .

DETD . . . this point on, the procedure was the same for both sets of controls. Cells were extracted with 30 .mu.l 10% **SDS**, 3 .mu.l Proteinase K (20 mg/ml) (Sigma, St. Louis, Mo.). The samples were incubated for 1 hour at 37.degree. C.,. . .

DETD . . . T4 kinase. Sample DNA aliquots and 16s DNA standards were prepared in 0.4 M NaOH (final volume 0.5 ml) and **boiled** for 10 min. Samples and standards were blotted onto Biotrans.TM. nylon membrane (ICN, Irvine, Calif.) using a slot blot apparatus. . . 80.degree. C. for 1 hour. The blot was prehybridized in 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 7% **SDS** for 1 hour at 37.degree. C. The .sup.32 P-labeled probe was added to the blot and incubated overnight. The blot. . . four times using a high stringency wash buffer (20 mM Tris-HCl, pH 7-8, 10 mM NaCl, 1 mM EDTA, 0.5% **SDS**, 37.degree. C.). The blot was dried, exposed to x-ray film and hybridization signals were quantitated by densitometry using a Visage. . .

DETD . . . exposure times (100.degree. C., 400 atm, 30 min) produced the greatest yield of DNA compared to that obtained by standard **SDS** lysis procedures. Genomic DNA appears to be completely unaffected by the extremes of temperature and pressure used in supercritical fluid. . .

DETD . . . recovery to 45%, while chloroform:methanol (1:1) pretreatment resulted in recovery of 78% of the DNA compared to that of the **SDS**-treated bacterial cultures. The differing recoveries may be due to differences in cell membranes compared to the other bacterial species (i.e.,. . .

DETD Samples of **Cryptosporidium** muris **oocysts** were prepared by filtration onto GF/F glass fiber filters. Each filter contained approximately 2.63.times.10.sup.7 **oocysts**. The filters were exposed to supercritical conditions of 400 atm at 100.degree. C. for 30 minutes and the nucleic acids. . .

L4 ANSWER 8 OF 14 USPATFULL

ACCESSION NUMBER: 2000:70449 USPATFULL

TITLE: GP900 glycoprotein and fragments for treatment and detection/diagnosis of **cryptosporidium**

INVENTOR(S): Petersen, Carolyn, Berkeley, CA, United States

PATENT ASSIGNEE(S): The Regents of The University of California, Oakland, CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 6071518		20000606
APPLICATION INFO.:	US 1997-928361		19970912 (8)

RELATED APPLN. INFO.: Continuation-in-part of Ser. No. US 1996-700651, filed on 14 Aug 1996 which is a continuation-in-part of Ser.

No. US 1995-415751, filed on 3 Apr 1995, now patented, Pat. No. US 5643772 which is a continuation of Ser. No. US 1993-71880, filed on 1 Jun 1993, now abandoned which is a continuation-in-part of Ser. No. US 1992-891301, filed on 29 May 1992, now abandoned

	NUMBER	DATE
PRIORITY INFORMATION:	US 1996-26062P	19960913 (60)

DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Mosher, Mary E.
LEGAL REPRESENTATIVE: Verny, Hana
NUMBER OF CLAIMS: 22
EXEMPLARY CLAIM: 1,5,13
NUMBER OF DRAWINGS: 12 Drawing Figure(s); 7 Drawing Page(s)
LINE COUNT: 3092
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

TI GP900 glycoprotein and fragments for treatment and detection/diagnosis of **cryptosporidium**

AB Peptides, polypeptides, glycoproteins, their functional mutants, variants, analogs, and fragments useful for treatment and detection/diagnosis of **Cryptosporidium** infections by competitive inhibition of the function of a **Cryptosporidium** protein/glycoprotein DNA and RNA encoding the **Cryptosporidium** protein/glycoprotein, mutants, variants and analogs and fragments thereof, and methods for production of recombinant or fusion proteins for use in. . .

SUMM . . . for treatment and detection/diagnosis of C. parvum infections. In particular, this invention concerns competitive inhibition of the function of a **Cryptosporidium** protein/glycoprotein comprised of a protein with or without carbohydrates attached thereto. Additionally, this invention concerns DNA and RNA encoding the **Cryptosporidium** protein/glycoprotein, mutants, variants and analogs and fragments thereof, and methods for production of recombinant or fusion proteins for use in. . .

SUMM The genus **Cryptosporidium** consists of Apicomplexan parasites that invade and develop within epithelial cells of the gastrointestinal, hepatobiliary and respiratory tracts of a wide variety of vertebrates including reptiles, birds and mammals. **Cryptosporidium** was recognized as a cause of animal disease for several decades before the first cases of human cryptosporidiosis were reported. . . the magnitude of disease caused by this parasite in both AIDS patients and immunocompetent hosts began to be appreciated. Subsequently, **Cryptosporidium** has been found to be one of the most common causes of human diarrhea worldwide, and to be an increasingly recognized cause of diarrhea in children, animal care workers, and travelers. (**Cryptosporidium** and Cryptosporidiosis, Ed. Fayer, R., CRC Press, Boca Raton (1997)).

SUMM Investigation of waterborne and nosocomial outbreaks uncovered a number of biological characteristics of **oocysts**. First, the infectious dose of a parasite is very low. The ID.sub.50 for human volunteers with normal immune systems is 132 **oocysts** (N. Engl. J. Med., 332:855 (1995)). Second, infected hosts, for example calves, excrete large numbers of **oocysts**, on the order of 10.sup.10 /day. Third, the **oocysts** are fully sporulated and ready to infect when excreted. Fourth, the **oocysts** are environmentally hardy. They remain infectious in cool, moist areas for 3-4 months. They are not killed by chlorine levels achievable in drinking water. Fifth, the **oocysts** are quite small, 4-6 .mu.m, and are thus difficult to filter.

SUMM U.S. Pat. No. 5,643,772 describes **Cryptosporidium** hybrid vector and transformed host cells specifically binding anti-**Cryptosporidium** antibodies. U.S. patent application Ser. No. 08/700,651 describes vaccines, antibodies, proteins, DNAs and RNAs for prophylaxis and treatment of **Cryptosporidium** parvum

infections. U.S. patent application Ser. No. 08/827,171 describes vaccines, antibodies and proteins for prophylaxis and treatment of **Cryptosporidium** parvum infections. The patent and both applications are hereby incorporated by reference.

SUMM During the development of the disease, infective forms of **Cryptosporidium**, called sporozoites and merozoites, appear to adhere to the host cell and release the contents of anterior organelles (rhoptries, micronemes. . .

SUMM . . . cryptosporidiosis as well as for detection of cryptosporidiosis in the environment. These methods also include techniques for reproducible propagation of **Cryptosporidium** protein/glycoprotein and for expression of **Cryptosporidium** protein/glycoprotein in large amounts, which antigen would provide a basis for production of competitive inhibitor molecules. In addition, these techniques. . . need to utilize synthetic molecules serving the same function as the protein competitive inhibitor. This approach requires that a specific **Cryptosporidium** protein/glycoprotein is cloned and identified as a potential candidate through its ability to competitively inhibit infection.

SUMM . . . method for treatment of cryptosporidiosis involving recombinant, engineered or otherwise modified protein, or substitutes which have the same function as **Cryptosporidium** GP900 protein/glycoprotein in competitively inhibiting infection. Additionally such proteins or substitutes would be used for detection/diagnosis of cryptosporidiosis through competitive. . .

SUMM . . . a means to detect the presence of GP900 for diagnosis in a human or animal host or for detection of **Cryptosporidium** in the environment.

SUMM Still another aspect of this invention concerns a DNA and RNA encoding the **Cryptosporidium** protein/glycoprotein and fragments thereof for use in production of the protein/glycoprotein for development of agents used for treatment and diagnosis/detection.

SUMM . . . this invention concerns a group of GP900 recombinant or expressed proteins which are targets of polyclonal antibodies, which proteins inhibit **Cryptosporidium** infection, invasion, or adhesion.

SUMM . . . C. parvum infection in a subject in need of such treatment, said method comprising administering to a subject infected with **Cryptosporidium** the peptide, polypeptide, glycoprotein, functional mutant, variant, analogue or fragment thereof formulated for delivery to the site of infection in amounts sufficient to competitively inhibit the **Cryptosporidium** at the site of infection.

SUMM Still yet another aspect of this invention is a method for treatment of **Cryptosporidium** infection comprising competitive inhibition of the function of GP900 resulting in the inhibition or prevention of infection of host cells. . .

SUMM Still yet another aspect of this invention is a method for prevention of **Cryptosporidium** infection of host cells resulting in cessation of the symptoms of infection, including but not limited to diarrhea.

SUMM Still yet another aspect of this invention concerns a method for diagnosing/detection of **Cryptosporidium** in a subject or in the environment by competitive inhibition, comprising steps:

SUMM . . . GP900 wherein the presence and the degree of receptor binding of GP900 of the invention indicates the presence of a **Cryptosporidium** organism in the subject or in the environment.

SUMM Still yet another aspect of this invention concerns a method for detecting anti-**Cryptosporidium** antibody in a subject, said method comprising steps:

SUMM . . . formation of antibody-antigen complex, wherein the presence of the complex inhibits GP900-receptor binding and thereby indicates the presence of a **Cryptosporidium** antibody in the subject.

SUMM Still another aspect of this invention is a **Cryptosporidium** diagnostic or detection kit comprising protein or glycoprotein according to the invention and a means for detection of native GP900. . .

DRWD FIG. 1 is an immunoblot of **Cryptosporidium** parvum **oocyst**/sporozoite proteins showing detection of the >900

sporozoite protein with monoclonal and polyclonal antibodies to GP900.

DRWD FIG. 2 shows the immunoprecipitation of .sup.125 I surface label **Cryptosporidium** parvum sporozoite proteins using monoclonal and polyclonal antibodies to GP900.

DRWD FIG. 5 is the immunoblot of **Cryptosporidium** parvum N-deglycosylated sporozoite/**oocyst** proteins using monoclonal antibodies to GP900.

DRWD FIG. 10 is a graphical illustration of the inhibition of parasite burden in vivo in neonatal mice challenged with **Cryptosporidium** and treated with oral anti-recombinant GP900 antibodies.

DRWD "Detection" means establishing or providing evidence for the presence or prior presence of living or dead **Cryptosporidium** by detecting protein or glycoprotein function or competition of function in the host, in a host tissue specimens, or in. . .

DRWD "Diagnosis" means establishment of the presence or prior presence of **Cryptosporidium** infection or disease by detecting protein or glycoprotein, protein or glycoprotein function or competition of function as a component of. . .

DRWD . . . at the surface of sporozoites or merozoites. GP900 is the target of antibodies which inhibit infection, invasion or adhesion of **Cryptosporidium**. GP900 includes protein and carbohydrate moieties attached to protein including variants defined by differential glycolysation and conformational changes.

DRWD . . . of antibodies to GP900 and to its described structural variants, such that the antibody inhibits infection, invasion or adhesion of **Cryptosporidium** through such interaction. These terms also mean the interaction of GP900 with host cells or receptors present on host cells such that GP900 prevents infection, invasion or adhesion of **Cryptosporidium** to the host cells.

DRWD "GP900 antigen" means a protein with or without a carbohydrate attached thereto which defines the capacity of **Cryptosporidium** sporozoites and merozoites to infect host cells.

DRWD "**Cryptosporidium** species" or **Cryptosporidium**" means any organism belonging to the genus **Cryptosporidium**, such as, for example, **Cryptosporidium** parvum or **Cryptosporidium** muris, but also includes currently less well characterized other organisms such as, for example, Cyclospora and it is also meant to include apicomplexan parasites which invade the gastrointestinal tract, such as Eimeria. **Cryptosporidium** species comprise Apicomlexan parasites which primarily invade cells of the gastrointestinal tract and cause disease in a susceptible host.

DETD . . . infection by competitive inhibition. The method for treatment according to the invention utilizes competitive inhibition of the function of a **Cryptosporidium** molecule comprised of a protein with or without carbohydrates attached thereto.

DETD This invention also provides DNA and RNA encoding the **Cryptosporidium** GP900 molecule, mutants, variants and analogs and fragments thereof, and methods for production of recombinant or fusion proteins for use. . .

DETD . . . detection and diagnosis of cryptosporidiosis in human and animal subjects by way of providing therapeutic compounds which competitively inhibit the **Cryptosporidium** or compounds which detect its existence using a principle of competitive inhibition. Recombinantly produced GP900, peptides, polypeptides, glycoproteins, and their functional mutants, variants, analogs, and fragments are produced for treatment, diagnosis and detection of infections caused by any **Cryptosporidium** organisms or any organism belonging to **Cryptosporidium** species.

DETD More specifically, the invention concerns identification of a **Cryptosporidium** protein/glycoprotein, comprised of a protein or polypeptide with or without a carbohydrate attached thereto, identification of DNA of the **Cryptosporidium** protein/glycoprotein gene, sequencing DNA encoding the molecule, and expressing portions of the locus encoding the **Cryptosporidium** protein/glycoprotein or their engineered analogues to prepare competitive inhibitor molecules for treatment/prophylaxis/detection/diag

nosis of infection in humans and animals.

DETD I. **Cryptosporidium** Protein/glycoprotein Antigens

DETD **Cryptosporidium** organisms and particularly **Cryptosporidium** parvum are coccidian parasites of the gastrointestinal tract that cause a clinical syndrome of diarrhea for which there is currently no effective treatment. Infectivity of **Cryptosporidium** is mediated by a protein or polypeptide molecules of sporozoites or merozoites, the infective forms of **Cryptosporidium**.

DETD During the development of this invention, it has been shown that a **Cryptosporidium** parvum expression library clone S34 encoded a portion of a protein larger than 900 kD, recognized by hyperimmune bovine colostrum. . .

DETD The GP900 protein was found to be highly abundant and easily visualized by Coomassie blue staining of proteins on **SDS**-polyacrylamide gels (**SDS**-PAGE) and is Triton X-100 soluble and N-glycosylated. The protein has been detected in micronemes of the invasive stages of **Cryptosporidium** by immunoelectronmicroscopy and has been shown to be accessible to surface radioiodination with .sup.125 I.

DETD . . . of six antibodies, namely 10C6, 7B3, and E6, made from a single fusion event in which the immunogen was an **oocyst** containing sporozoites, were specific to GP900, suggesting that GP900 is a highly immunogenic molecule of sporozoites. Three of eight antibodies,. . .

DETD The DNA encoding **Cryptosporidium** antigen can be coupled to **Cryptosporidium** DNA encoding regulatory elements located downstream or upstream or on another chromosome in the **Cryptosporidium** genome. These operably coupled DNA segments are able to bind selectively and specifically to **Cryptosporidium** molecules, such as proteins.

DETD A. Identification of Protein GP900 as **Cryptosporidium** Antigen

DETD **Cryptosporidium** antigen identified as GP900 protein is a high molecular weight glycoprotein of a Mr greater than 900 kilodaltons (kD). The. . . molecular weight of approximately 150-250 kD. The GP900 protein has been identified as a target of anti-GP900 antibodies which inhibit **Cryptosporidium** infection, invasion or adhesion.

DETD GP900 proteins were identified and isolated from **oocysts** of the Iowa, AUCP-1 NINC isolates of **Cryptosporidium** parvum, as described in Example 1 and tested for their interaction with specific anti-GP900 antibodies. Proteins which were shown to. . .

DETD Polyclonal antibodies against **SDS** solubilized GP900 and MAb 10C6, prepared according to Example 4, which were previously shown to detect GP900, were used for detection of molecular species which are immunoprecipitable with both mono and polyclonal antibodies. A Western blot probe of **oocyst**/sporozoite proteins is seen in FIG. 1. Immunoprecipitation of sporozoite surface labeled proteins with mono and polyclonal antibodies as seen in. . .

DETD FIG. 1 shows an immunoblot of **Cryptosporidium** parvum **oocyst**/sporozoite proteins of the AUCP-1 isolate separated by **SDS**-PAGE. FIG. 7, Lane 1 shows the MAb 10C6 culture supernatant, Lane 2 shows the polyclonal anti-GP900 in 1:5000 dilution.

DETD FIG. 2 shows immunoprecipitation of .sup.125 I radiolabelled **Cryptosporidium** parvum sporozoite surface proteins of the AUCP-1 isolate separated by 5-15% **SDS**-PAGE. FIG. 2, lane 1 shows radiolabeled **Cryptosporidium** parvum sporozoite surface protein control (10.sup.7 sporozoites/lane). Lane 2 shows radiolabeled **Cryptosporidium** parvum sporozoite surface proteins immunoprecipitated with polyclonal anti-GP900.

DETD . . . sporozoites. Polyclonal anti-GP900 antibody is thus an appropriate antibody for GP900 localization experiments and for detection of clones in a **Cryptosporidium** expression library.

DETD In order to determine whether GP900 similarly was shed from the surface of **Cryptosporidium** sporozoites, living sporozoites were allowed to glide on poly-L-lysine coated slides as described in Example 3. Results are shown in. . .

DETD . . . the sporozoites with formaldehyde. MAb 7B3 was used because it

was previously shown to detect only GP900 on immunoblots of **Cryptosporidium** sporozoite proteins. FIG. 3 shows that GP900 is present around the living sporozoite and is shed from the posterior aspect. . .

DETD . . . particles of the second antibody, used to localize GP900 polyclonal antibodies, are concentrated. GP900 was also seen in sporozoites within **oocysts** (data not shown). The rhoptries and dense granules were not labeled. No surface labeling of sporozoites and merozoites was observed.. . .

DETD In order to show that GP900 is a glycoprotein, N-linked carbohydrate was enzymatically removed from **Cryptosporidium** parvum **oocyst**/sporozoite proteins and the remaining protein was separated by **SDS**-PAGE and detected with MAb 10C6 on an immunoblot by the techniques described in Infect. Immun., 60:5132 (1992) and in Example. . .

DETD An anti-GP900 polyclonal antibody, affinity purified from **oocyst** /sporozoite antibodies on the protein expressed by a gt11 clone S34 using S34 recombinant eluted antibody (S34 REA), also detected a. . .

DETD The GP900 gene of **Cryptosporidium** parvum was isolated from a naturally infected neonatal calf (NINC) isolate. DNA from the calf isolate was used to prepare. . .

DETD FIG. 6 shows that the DB8 probe of the NINC **Cryptosporidium** isolate hybridizes to a single DNA fragment in EcoRI, Bgl II and Hind III digests of the Iowa strand, indicating. . .

DETD The presence of abundant cysteines on a surface protein of **Cryptosporidium** which is functionally homologous to the circumsporozoite protein of malaria strongly suggests that these cysteines participate in binding phenomena and. . .

DETD . . . vector pTrusFux according to supplier's protocols (Invitrogen). Sense and anti-sense PCR amplification oligonucleotides, which allowed the amplification from Iowa genomic **Cryptosporidium** DNA of domain 1 or domain 3 with Kpn 1 and Xba I sequences at the 5' and 3' ends. . .

DETD . . . proteins were prepared according to Example 5. These various antibody preparations were used to probe an immunoblot of proteins from **Cryptosporidium** parvum **oocysts**/sporozoites as described in Example 7. Results are shown in FIG. 7.

DETD FIG. 7 is an immunoblot of proteins obtained from **Cryptosporidium** parvum **oocysts** or sporozoites. Marker size in kD is indicated. Lane 1 contains **oocyst**/sporozoite proteins probed with pre-immune rabbit serum. Lanes 2-4 are probed with the serum of rabbit immunized with recombinant S34 and. . .

DETD . . . shows that the pre-immune serum from the rabbit which received the S34 antigen is mildly reactive to two proteins of **Cryptosporidium** parvum. After immunization with the S34 antigen (lanes 2-4), the antisera react with a whole variety of proteins including GP900,. . .

DETD In order to determine whether the native or recombinant antibodies raised against **Cryptosporidium** antigen GP900 or a fraction thereof are able to inhibit **Cryptosporidium** infection in vivo, the anti-S34-.beta.-galactosidase antibodies were tested in a neonatal mice model as described in Example 16. Results are. . .

DETD FIG. 10 is a graph representing the amount of excretion of **Cryptosporidium oocysts** per day in mice treated with phosphate buffered saline (bar 1); anti-.beta.-galactosidase (bar 2); anti-Ag4.beta.-galactosidase (bar 3); anti-S34-.beta.-galactosidase (bar 4);. . .

DETD As seen in FIG. 10, anti-S34 (bar 4) reduced the **oocysts** shed by about 50% relative to control PBS (bar 1) and anti-.beta.-galactosidase antibody (bar 2). Although crude antisera was used,. . . 40529 (bar 5), the positive control antibody which had previously been shown to prevent cryptosporidial disease in calves challenged with **Cryptosporidium** (Infect. Immun., 61:4079-4084 (1993)).

DETD From the results obtained in these experiments, it is clear that clone S34 encodes a **Cryptosporidium** antigen and that the antibodies specifically raised against this antigen are able to inhibit

Cryptosporidium infection in vivo.

DETD . . . .beta.-galactosidase, Ag4-.beta.-galactosidase and S34-.beta.-galactosidase, as described in Example 14. In this assay, the same magnitude of inhibition of adhesion of **Cryptosporidium** sporozoites to Caco-2 cells (mean O.D. 50% of control in ELISA) with a 1:50 dilution of anti S34-.beta.-galactosidase was conferred. . .

DETD In addition, these results were comparable to those seen when a 1:100 dilution of anti-**Cryptosporidium** murine ascites (48% inhibition) a polyclonal rabbit anti-**Cryptosporidium** antiserum (inhibition 51%) were previously assayed in this system (data not shown). Similarly to the in vivo model, in this. . . Additionally, these results show that antibodies to recombinant GP900 correlate significantly with the inhibitory activity of HBC Ig 40529 and anti-**Cryptosporidium** antibodies from mouse and rabbit sources.

DETD Thus antibodies against the recombinant S34 protein are able to significantly inhibit **Cryptosporidium** infection in vitro and in vivo indicating the usefulness of the anti-S34 antibody for both anti-**Cryptosporidium** prophylaxis and therapy of a human or animal host.

DETD Purified native GP900 of **Cryptosporidium** has recently been found to bind to Caco-2A cells in a saturable, dose dependent manner in an assay designed to. . .

DETD A method for therapeutic treatment, retardation, or inhibition of **Cryptosporidium** infection comprises administering to a subject in need of such treatment an amount of a recombinant GP900 protein, or an engineered analogue thereof, prepared according to the invention, effective to inhibit the existing clinically apparent **Cryptosporidium** infection.

DETD A method of prophylaxis of **Cryptosporidium** infection comprises administering to a subject in need of such treatment an amount of a recombinant protein, or an engineered analogue thereof, prepared according to the invention, effective to provide protection against the invasion of **Cryptosporidium** and establishment of clinical infection.

DETD In AIDS patients **Cryptosporidium** parvum may cause a devastating disease for which there is no treatment. Understanding of the organism and the pathophysiology of the disease it produces, and development of treatment, are very important steps in the treatment of **Cryptosporidium** infection.

DETD . . . and sense and antisense for domain 1 and domain 3 (SEQ ID NOs. 21-24) were used to amplify the Iowa **Cryptosporidium** DNA. The sense primers have a KpN1 site and the antisense primers have an XbaI site engineered into the 5'. . .

DETD An important part of this invention is a method of diagnosing **Cryptosporidium** infection or detection of **Cryptosporidium** in the tissue samples or in the environment. The detection method for environmental samples comprises contacting such a sample with the invention for purposes of detecting **Cryptosporidium**.

DETD The diagnostic method comprises contacting a body fluid or tissue with the invention for purposes of detecting the presence of **Cryptosporidium**.

DETD Qualitative and Quantitative Detection of **Cryptosporidium** Formulations and Kits

DETD For qualitative and quantitative determination of the presence of the **Cryptosporidium** infection and environmental contamination, a kit for the diagnosis/detection of **Cryptosporidium** is used. The kit comprises the peptide, polypeptide, glycoprotein, functional mutant, variant, analogue or fragment thereof and a means for. . .

DETD The kit is utilized for the detection of endogenous antibodies/antigens/DNA/RNA produced by a subject that is afflicted with cryptosporidiosis and **Cryptosporidium** antigens/DNA/RNA present in the environmental samples. Even at the early stages where the parasite is commencing invasion of a subject's cells, some amount of the **Cryptosporidium** antigen or the specific antibody detectable in serum. The kit detects either the antigen with the polyclonal antibodies

or the presence of the anti-**Cryptosporidium** antibody with the antigen. The complexing immunoreaction is detected by staining, radiography, immunoprecipitation or by any other means used in. . .

DETD The current invention provides means for suitable detection or diagnosis of **Cryptosporidium** parvum species and isolates and cryptosporidiosis or, for a therapeutic use of recombinant or otherwise modified or engineered variants, analogues. . .

DETD **Cryptosporidium** parvum Parasites

DETD This example illustrates the protocol used for isolation of **Cryptosporidium** parvum parasites.

DETD **Oocysts** of the Iowa, NINC and AUCP-1 isolates of **Cryptosporidium** parvum were passaged through neonatal calves at the Animal Resources Services, University of California, Davis or obtained from a commercial source (Pat Mason) and the **oocysts** were purified and encysted. The detailed protocol is described in Infect. Immun., 61:4079 (1993). **Oocysts** containing sporozoites were solubilized, resolved by **SDS**-PAGE and subjected to immunoblotting, according to Infect. Immun., 60:5132 (1992).

DETD 10 week-old female BALB/c mice were immunized four times intraperitoneally with approximately 5.times.10.sup.5 sonicated 10.sup.5 **Cryptosporidium** parvum **oocysts**. The polyclonal antibody fraction of the ascites which was shown to react with the **Cryptosporidium** parvum sporozoite surface, the **oocyst** surface, and/or with internal antigens of the **oocysts**, was assessed by an IFA as described in Infect. Immun., 60:5132 (1992).

DETD For monoclonal antibody production, mice treated as above were immunized intravenously with the supernatant from sonicated **Cryptosporidium** parvum **oocysts** three days before fusion as described in J. Immunol., 123:1548 (1979) and J. Parasitol., 68:1029 (1982). Hybridoma supernatants were used. . .

DETD In order to determine whether GP900 was shed by the **Cryptosporidium** sporozoite in the absence of a specific antibody, living sporozoites were allowed to glide on poly-L-lysine coated microscopic slides. Slides. . .

DETD The Triton X-100 (1%) soluble fraction of 2.times.10.sup.8 **oocysts** was immunoprecipitated with MAb10C6. A >900 kD MW species was identified in gels stained with Coomassie blue in water and excised. Frozen gel containing 2.times.10.sup.7 **oocyst** /sporozoites was pulverized and emulsified in 150 .mu.l PI of PBS and 150 .mu.l complete Freund's adjuvant (CFA) for intraperitoneal (IP). . .

DETD . . . in 200 .mu.l of 2M Tris, pH 7.4. All affinity purified antibodies reacted with the fusion protein and the respective **Cryptosporidium** protein but not other E. coli proteins.

DETD Polyclonal rabbit antisera from an unimmunized rabbit was evaluated for reactivity against **Cryptosporidium** antigens at a 1:1000 dilution on immunoblot and found to be free of reactivity. One ml of polyclonal rabbit antisera,. . . Tris, pH 6.0. Antibody concentration was determined by absorbance at 280 nm and integrity of the Ig was verified by **SDS**-PAGE. Positive control antibody, HBC Ig 40529 has been previously described in Infect. Immunol., 61:(10); 4079 (1993).

DETD **Oocysts** (10.sup.6 lane) were solubilized in denaturing sample buffer containing 5% .beta.ME (.beta.-mercaptoethanol), resolved by **SDS**-PAGE and subjected to immunoblotting according to Infect. Immunol., 60:532 (1992). Proteins were visualized after incubation with primary antibody with .sup.125. . .

DETD DNA was purified from 1.times.10.sup.9 **Cryptosporidium** parvum **oocysts** as described in Example 1. DNA was digested with the restriction enzymes according to procedures provided by the manufacturer Promega.. . .

DETD mRNA was purified from MDCK cells, or MDCK cells infected with sporozoites at a ratio of 1 **oocyst**/1 MDCK cell, harvested at 24 and 48 hours using guanidinium thiocyanate and oligo-dT cellulose isolation (Ambion mRNA purification kit, Albion,. . .

DETD Surface Radioiodination and Immunoprecipitation of **Cryptosporidium** Sporozoite Proteins

DETD This example describes the methods used for surface radio-iodination and immunoprecipitation of **Cryptosporidium** sporozoite proteins.

DETD **Oocysts** were bleached, encysted and separated from sporozoites prior to iodination of the sporozoite surface and immunoprecipitation of surface proteins as. . .

DETD . . . 0.5% Triton X-100, pH 7.4) at 100,000.times.g for 1 hour at 40.degree. C. An aliquot of membrane proteins in 2% **SDS** 5% p-sample buffer was prepared for total sporozoite surface protein analysis. Aliquots of membrane proteins extracted in 2% **SDS** were diluted with 9 volumes NETT plus 1% high quality bovine serum albumin (BSA) obtained from Sigma; 1 volume 1%. . . overnight incubation. Protein A Sepharose 4B beads were added to immobilize the immunoprecipitated proteins. Parasite proteins were solubilized in 2% **SDS** sample buffer containing .beta.-mercaptoethanol. Samples were **boiled** 5 minutes and separated by 5-15% gradient **SDS**-PAGE.

DETD Immunoelectronmicroscopic Localization of GP900 in **Cryptosporidium** parvum-Infected Rat Intestinal Tissue

DETD This example describes the immunoelectronmicroscopic methods used for localization of GP900 antigen in **Cryptosporidium** parvum infected rat intestinal tissue.

DETD **Oocysts** were used to inoculate confluent Madin Darby Canine Kidney (MDCK) cell monolayers for in vitro inhibition assays of sporozoite invasion. . . chamber slides from Nunc Inc., Napersville, Ill., containing 10.sup.5 MDCK cells were overlaid with 400 l RPMI medium containing 1.5.times.10.sup.5 **oocysts** and antibody or colostrum samples to be tested for inhibitory capacity. Each experimental data point was an average of the. . .

DETD Controls included hyperimmune bovine colostrum 40529 Ig (HBC Ig) raised against **Cryptosporidium oocysts** and sporozoites and SHAM-HBC raised against a herd vaccine at ImmuCell Corp, Portland, Me.

DETD . . . in Example 12. The antibody, at concentrations of 10, 50, 100 and 500 .mu.g/ml in RPMI, was incubated with encysted **oocysts** on MDCK cell monolayers for two hours. The wells were washed out and refilled with RPMI.

DETD Control wells contained equal amounts of **oocysts** and RPMI alone, S34-GST at 100 nM, anti-**oocyst**/sporozoite antibody at a 1:40 dilution and HBC Ig 40529 at a 1:40 dilution. As described in FIG. 9 invasion/intracellular development. . .

DETD Inhibition of **Cryptosporidium** Invasion and Intracellular Development in MDCK cells with MAb 10C6

DETD This example describes studies performed to detect inhibition of **Cryptosporidium** invasion and intracellular development in vitro using monoclonal antibodies.

DETD **Cryptosporidium oocysts** of the AUCP-1 isolate were encysted and three sporozoite monoclonal antibodies, MAb 10C6, 7B3 and E6, were prepared as described. . .

DETD . . . and intracellular development were found to be inhibited by >95% compared to the control antibody. Sequential observation of viable, unfixed **Cryptosporidium** sporozoites by differential phase contrast microscopy after addition of MAb 10C6 revealed initial reactivity of the MAb with the surface. . .

DETD Iowa **oocysts** (5.times.10.sup.8) were excysted at 37.degree. C. for two hours and pelleted at 4,000.times.g for 10 minutes at 4.degree. C. The. . . 10 mM, PMSf 2 mM. The supernatant was concentrated by ultrafiltration to 350 .mu.l (14.2.times.) (Centricon 10, Amicon). Silver stained **SDS**-PAGE gel of 10 and 20 .mu.l aliquots revealed equal amounts of 47 kD, 120 kD and >900 kD proteins. The. . .

DETD In vivo Inhibition of **Cryptosporidium** Infection in Mice Challenged with **Cryptosporidium Oocysts** with Anti-S34-.beta.-Galactosidase and anti-antigen for .beta.-Galactosidase Polyclonal Antibodies

DETD This example describes the method used for determination of the in vivo inhibition of **Cryptosporidium** infection of mice challenged with **Cryptosporidium oocysts** and treated with specific anti-S34-.beta.-galactosidase and anti-antigen

4-.beta.-galactosidase (FIG. 10) polyclonal antibodies.

DETD Anti-.beta.-galactosidase, anti-S34-.beta.-galactosidase, anti-Ag4-.beta.-galactosidase and HBC Ig 40529 were tested for inhibitory activity in a neonatal mouse model of **Cryptosporidium** infection using GCH-1 obtained from an NIH repository.

DETD Three experiments were performed and the data pooled. In each experiment 5 neonatal mice per group were infected with **Cryptosporidium** and were fed either 20 .mu.l control PBS, 20 .mu.l of the 3 rabbit antibodies or 20 .mu.l of a. . . human AIDS patients for cryptosporidiosis, was given to mice in 2 experiments. Infection was scored as the mean number of **oocysts** shed per day during a 5 day collection period.

DETD . . . experiment was researched in which antibodies were assayed in vivo in groups of 7 CDI neonatal mice challenged with 10.sup.4 **oocysts** on day 6. **Oocyst** output was scored in Sheather's solution and is expressed as 10.sup.5 /ml. Antisera were diluted 1:2 in 50 mM NaHCO3. FIG. 10 is a graph representing the amount of excretion of **Cryptosporidium oocysts** per day in mice treated with phosphate buffered saline (bar 1); anti-.beta.-galactosidase (bar 2); anti-Ag4-.beta.-galactosidase (bar 3); anti-S34-.beta.-galactosidase (bar 4);. . . 1:5 HBC Ig 40529 (bar 5); and paromomycin (bar 6). As seen in FIG. 10, anti-S34 (bar 4) reduced the **oocysts** shed by about 50% relative to control PBS (bar 1) and anti-.beta.-galactosidase antibody (bar 2).

DETD . . . the domain 1 sequence. When used as a pair of PCR amplification oligonucleotides, these oligonucleotides allowed the amplification from genomic **Cryptosporidium** DNA of the entire domain 1 and domain 3 sequences with Kpn 1 and Xba I sequences at the 5'. . .

CLM What is claimed is:

1. A fragment of the GP900 protein of **Cryptosporidium** parvum, wherein the fragment comprises domain 1, 2, 3, 4, or 5.

5. A fragment of the GP900 protein of **Cryptosporidium** parvum, wherein the fragment competitively inhibits sporozoite or merozoite attachment or invasion in cultured cells, or inhibits specific binding of. . .

13. A fragment of the GP900 protein of **Cryptosporidium** parvum wherein the fragment is capable of inducing the production of antibodies which ameliorate parasite infection when said antibodies are. . .

. . . producing antibodies capable of inhibiting or ameliorating parasite attachment/invasion, comprising administering to a suitable host a purified GP900 protein of **Cryptosporidium** parvum, or the fragment of any of claims 5-11.

22. A method of treatment or prophylaxis of **Cryptosporidium** infections by competitive inhibition comprising steps: a) preparing antibodies capable of inhibiting or ameliorating parasite attachment or invasion, using a purified GP900 protein of **Cryptosporidium** parvum or the fragment of any of claims 5-11; and b) administering said antibodies to a subject in need of. . . or prophylaxis, wherein said antibodies competitively inhibit binding of a GP900 ligand protein or attachment or invasion of cells with **Cryptosporidium**.

L4 ANSWER 9 OF 14 USPATFULL

ACCESSION NUMBER: 2000:7383 USPATFULL

TITLE: Vaccines, antibodies, proteins, glycoproteins, DNAs and RNAs for prophylaxis and treatment of **Cryptosporidium** parvum infections

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CAS INDEXING IS AVAILABLE FOR THIS PATENT.

TI Vaccines, antibodies, proteins, glycoproteins, DNAs and RNAs for prophylaxis and treatment of **Cryptosporidium** parvum infections

AB Vaccines, antibodies, proteins, glycoproteins, DNAs and RNAs useful for passive or active prophylaxis and treatment of **Cryptosporidium** infections. **Cryptosporidium** antigen comprised of a protein with or without carbohydrates attached thereto. Polyclonal and monoclonal antibodies directed against the antigen. DNA and RNA encoding the **Cryptosporidium** antigen, mutants, variants and fragments thereof.

SUMM This invention concerns vaccines, antibodies, proteins, glycoproteins, DNAs and RNAs for prophylaxis and treatment of **Cryptosporidium** or **Cryptosporidium** infections. In particular, this invention concerns a **Cryptosporidium** antigen comprised of a protein with or without carbohydrates attached thereto, as well as polyclonal and monoclonal antibodies directed against the antigen. Additionally, the invention concerns DNA and RNA encoding the **Cryptosporidium** antigen, mutants, variants and fragments thereof.

SUMM The genus **Cryptosporidium** consists of Apicomplexan parasites that invade and develop within epithelial cells of the gastrointestinal, hepatobiliary and respiratory tracts of a wide variety of vertebrates including reptiles, birds and mammals. **Cryptosporidium** was recognized as a cause of animal disease for several decades before the first cases of human cryptosporidiosis were reported. . . magnitude of the disease caused by this parasite in both AIDS patients and immunocompetent hosts began to be appreciated. Subsequently, **Cryptosporidium** has been found to be among the top four causes of human diarrhea worldwide, and to be an increasingly recognized cause of diarrhea in children, animal care workers, and travelers. (**Cryptosporidium** in Humans, Ed. J. P. Dubai et al., CRC Press, Boca Raton (1990)).

SUMM Waterborne and nosocomial spread reveal a number of biological characteristics of **oocysts**. First, the infectious dose of the parasite is very low. The ID.sub.50 for human volunteers with normal immune systems is 132 **oocysts** (New Engl. J. Med., 332:855 (1995)). Second, infected hosts, for example calves, excrete large numbers of **oocysts**; on the order of 10.sup.10 /day. Third, the **oocysts** are fully sporulated and ready to infect when excreted. Fourth, the **oocysts** are environmentally hardy; they remain infectious in cool, moist areas for 3-4 months, and they are not killed by chlorine levels permissible in drinking water. Fifth, the **oocysts** are quite small, 4-6 .mu.m, and are thus difficult to filter.

SUMM The infective forms of **Cryptosporidium**, called sporozoites and merozoites, adhere to the host cell and release the contents of anterior organelles (rhoptries, micronemes or dense. . .

SUMM While the actual interaction between **Cryptosporidium** and the

host's immune system is poorly understood, it is known that disruption of either the cellular or the humoral. . . 8:24 (1992)). However, specific antibodies alone neutralize the organism's infectivity. In vitro and in vivo observations indicate that antibodies to **Cryptosporidium** parvum inhibit invasion and intracellular development leading to protection in challenge experiments, or amelioration of infection in established disease (Infect.. . .

SUMM One source of such antibodies is hyperimmune bovine colostrum (HBC) collected from cows immunized with **Cryptosporidium oocysts**. Calves challenged with **Cryptosporidium oocysts** are protected from the development of the disease by the administration of HBC (Infect. Immun., 61:4079 (1993)). Some immunocompromised AIDS patients infected with **Cryptosporidium** have also responded to HBC with a reduction in, or disappearance of, the symptoms of the disease (Gastroenterology, 98:486 (1990)).. . . and/or develop intracellularly in vitro and it has been used to immunoprecipitate at least 22 different surface radioiodinated proteins of **Cryptosporidium** sporozoites. Western blot analysis of proteins of whole **oocysts**, which contain sporozoites, indicates that HBC predominantly recognizes two proteins of sizes 250 kDa and >900 kDa (Infect. Immun., 61:4079. . .

SUMM The use of HBC for human use is problematic. HBC produced using whole **oocysts** is batch dependent and this may lead to the development of passive immune preparations which are not uniform in immunogenicity. . . would be desirable to allow preparation of large amounts of antigen expressed in heterologous systems rather than to purify the **oocyst**.

SUMM Additionally, there is a need to have available methods for reproducible expression of specific targets for **Cryptosporidium** antigens in large amounts, which antigens would provide a better immunogen. This approach requires that specific **Cryptosporidium** antigen genes are cloned and identified as potential candidates through their ability to elicit an antibody response that is immunoprotective.. . . to humans or animals, testing in in vitro assay of its inhibitory effect on invasion or intracellular development of the **Cryptosporidium** organism in cultured cells, and in vivo studies, would be desirable.

SUMM . . . of cryptosporidiosis and to express a portion of the GP900 sequence/locus to provide target protein antigens allowing production of recombinant anti-**Cryptosporidium** vaccines and passive immune products.

SUMM One aspect of this invention concerns vaccines, antigens, antibodies, proteins, glycoproteins, DNAs and RNAs for prophylaxis and treatment of **Cryptosporidium** or **Cryptosporidium** infections.

SUMM Another aspect of this invention concerns a **Cryptosporidium** antigen comprised of an immunogenic protein without attached carbohydrates.

SUMM Another aspect of this invention concerns a **Cryptosporidium** antigen comprised of an immunogenic protein with attached carbohydrates.

SUMM Another aspect of this invention concerns polyclonal or monoclonal antibodies directed against the **Cryptosporidium** antigen.

SUMM Another aspect of this invention concerns DNA and RNA encoding or representing the **Cryptosporidium** antigen and fragments thereof.

SUMM . . . concerns polyclonal or monoclonal antibodies directed against invasive stages of cryptosporidial species capable of preventing and ameliorating the invasion of **Cryptosporidium** infection.

SUMM Another aspect of this invention concerns a synthetic or recombinant vaccine, useful for active immunization of animals and humans against **Cryptosporidium** infection.

SUMM . . . aspect of this invention concerns a synthetic or recombinant DNA vaccine, capable of endogenous development of an inhibitory amount of anti-**Cryptosporidium** parvum antibodies.

SUMM . . . aspect of this invention concerns a synthetic or recombinant RNA vaccine, capable of endogenous development of an inhibitory amount of anti-**Cryptosporidium** parvum antibodies.

SUMM Another aspect of this invention concerns a method for prophylaxis and

treatment of **Cryptosporidium** or **Cryptosporidium** infections using vaccines, antibodies, proteins, glycoproteins, DNAs and RNAs of the invention.

SUMM Another aspect of this invention concerns a method of prophylaxis, treatment, inhibition or retardation of a **Cryptosporidium** infection, comprising administering to a subject in need of such treatment an amount of anti-**Cryptosporidium** polyclonal or monoclonal antibodies, prophylactically or therapeutically effective, to provide immunity against infection or treatment for the disease.

SUMM Another aspect of this invention concerns a method of prophylaxis, treatment, retardation, or inhibition of **Cryptosporidium** infection, comprising administering to a subject in need of such treatment, a vaccine containing the polypeptide or glycoprotein of this invention or its DNA or RNA, capable of endogenous stimulation of the production of an inhibitory amount of anti-**Cryptosporidium** antibodies.

DRWD FIG. 1 is an immunoblot of **Cryptosporidium** parvum **oocyst**/sporozoite proteins showing detection of the >900 sporozoite protein with monoclonal and polyclonal antibodies to GP900.

DRWD FIG. 2 is the immunoprecipitation of .sup.125 I surface label **Cryptosporidium** parvum sporozoite proteins using monoclonal and polyclonal antibodies to GP900.

DRWD FIG. 4 is the immunoblot of **Cryptosporidium** parvum N-deglycosylated sporozoite/**oocyst** proteins using monoclonal antibodies to GP900.

DRWD FIG. 10 is a graphical illustration of the inhibition of parasite burden in vivo in neonatal mice challenged with **Cryptosporidium** and treated with oral anti-recombinant GP900 antibodies.

DRWD FIG. 11 is an immunoblot of **Cryptosporidium** parvum **oocyst**/sporozoite proteins using polyclonal antibodies to P68.

DRWD FIG. 13 is a graphical illustration of the inhibition of parasite burden in vivo in neonatal mice challenged with **Cryptosporidium** and treated with oral anti-recombinant P68 antibodies.

DETD . . . at the surface of sporozoites or merozoites. GP900 is the target of antibodies which inhibit infection, invasion or adhesion of **Cryptosporidium**.

DETD . . . Mr between approximately 50 and 100 kilodaltons which is a target of antibodies which inhibit infection, invasion or adhesion of **Cryptosporidium**.

DETD . . . the interaction of antibodies to GP900 and structural variants described, such that the antibody inhibits infection, invasion or adhesion of **Cryptosporidium**.

DETD . . . the interaction of antibodies to P68 and structural variants described, such that the antibody inhibits infection, invasion or adhesion of **Cryptosporidium**.

DETD "GP900 antigen" means a protein with or without a carbohydrate attached thereto which defines the capacity of **Cryptosporidium** sporozoites and merozoites to infect host cells.

DETD "P68 antigen" means a protein with or without a carbohydrate attached thereto which defines the capacity of **Cryptosporidium** sporozoites and merozoites to infect host cells.

DETD "**Cryptosporidium** species" means any organism belonging to the genus **Cryptosporidium**, such as, for example, **Cryptosporidium** parvum or **Cryptosporidium** muris, but also includes currently less well characterized other organisms such as, for example, Cyclospora and it is also meant to include apicomplexan parasites which invade the gastrointestinal tract, such as Eimeria. **Cryptosporidium** species comprise Apicomlexan parasites which primarily invade cells of the gastrointestinal tract and cause disease in a susceptible host.

DETD . . . invention relates to vaccines, antibodies, proteins, glycoproteins, DNAs and RNAs useful for prophylaxis and treatment of infections caused by any **Cryptosporidium** organism or any organism belonging to the **Cryptosporidium** species.

DETD More specifically, the invention concerns: identification and isolation of **Cryptosporidium** antigens comprised of a protein or

polypeptide with or without a carbohydrate attached thereto; identification of the DNA of the **Cryptosporidium** antigen gene within the locus; sequencing DNA encoding **Cryptosporidium** antigens; expressing portions of the locus encoding the **Cryptosporidium** antigens; and using the expressed antigens to prepare vaccines or polyclonal or monoclonal antibodies.

DETD I. **Cryptosporidium** Antigens

DETD **Cryptosporidium** organisms and particularly **Cryptosporidium** parvum are coccidian parasites of the gastrointestinal tract that cause a clinical syndrome of diarrhea for which there is currently no effective treatment. Infectivity of **Cryptosporidium** is mediated by a specific protein or polypeptide antigens of sporozoites or merozoites, the infective forms of **Cryptosporidium**.

DETD The DNA encoding **Cryptosporidium** antigen can be coupled to **Cryptosporidium** DNA encoding regulatory elements located downstream or upstream or on another chromosome in the **Cryptosporidium** genome. These operably coupled DNA segments are able to bind selectively and specifically to **Cryptosporidium** molecules, such as proteins.

DETD . . . this invention, it has been shown and described in Infect. Immun., 60:2343 (1992), 60:5132 (1992), and 61:4079 (1993), that a **Cryptosporidium** parvum expression library clone S34 encoded a portion of a protein larger than 900 kDa, recognized by hyperimmune bovine colostrum. . .

DETD The GP900 protein is highly abundant and is easily visualized by Coomassie blue staining of proteins on **SDS**-polyacrylamide gels (**SDS**-PAGE). Furthermore, it is known to be Triton X-100 soluble and N-glycosylated.

DETD . . . of six antibodies, namely 10C6, 7B3, and E6, made from a single fusion event in which the immunogen was an **oocyst** containing sporozoites, were specific to GP900, suggesting that GP900 is a highly immunogenic molecule of sporozoites. Three of eight antibodies,. . .

DETD The second **Cryptosporidium** antigen is a smaller protein identified as P68.

DETD A. **Cryptosporidium** Antigen Protein GP900

DETD **Cryptosporidium** antigen GP900 is a high molecular weight glycoprotein of a Mr greater than 900 kilodaltons (kDa). The GP900 protein was. . . molecular weight of approximately 150-250 kDa. The GP900 protein has been identified as a target of anti-GP900 antibodies which inhibit **Cryptosporidium** infection, invasion or adhesion.

DETD **Cryptosporidium** parvum was identified and isolated from **oocysts** of the Iowa and AUCP-1 isolates of **Cryptosporidium** parvum passaged through neonatal calves, as described in Example 1. **Oocysts** containing sporozoites were solubilized, resolved by **SDS**-PAGE and subjected to immunoblotting, according to Infect. Immun., 60:5132 (1992). Proteins which are targets of an anti-**oocyst**/sporozoite antibody were visualized after incubation with the primary antibody by enzyme-linked immunosorbent assay (ELISA) or with .sup.125 I labeled Protein. . .

DETD . . . development of this invention, found to react predominantly with two proteins above 200 kDa in a Western blot of solubilized **oocyst**. The first protein had a size of 250 kDa. The second protein was >900 kDa and comigrated with GP900. In an attempt to determine whether the 250 kDa protein is a component of GP900, polyclonal antibodies against **SDS** solubilized GP900 were prepared.

DETD Identification of the GP900 protein from the **oocyst** of Iowa and AUCP-1 isolates is illustrated in FIGS. 1 and 2.

DETD Visualized **Cryptosporidium** antigen proteins were surface radioiodinated and immunoprecipitated using the method described in Infect. Immun., 61:4079 (1993).

DETD . . . was identified and excised, and used for immunization of mice for production of the anti-GP900 antibody. Polyclonal antibodies prepared against **SDS** solubilized GP900 and MAb 10C6, which were previously shown to detect GP900, were used to probe a Western

blot, as. . .

DETD FIG. 1 shows a immunoblot of **Cryptosporidium** parvum **oocyst**/sporozoite proteins of the AUCP-1 isolate separated by **SDS**-PAGE. Lane 1 shows the MAb 10C6 culture supernatant. Lane 2 shows the polyclonal anti-GP900 in 1:5000 dilution.

DETD FIG. 2 shows immunoprecipitation of .sup.125 I radiolabeled **Cryptosporidium** parvum sporozoite surface proteins of the AUCP-1 isolate separated by 5-15% **SDS**-PAGE. Lane 1 shows radiolabeled **Cryptosporidium** parvum sporozoite surface protein control (107 sporozoites/lane). Lane 2 shows radiolabelled **Cryptosporidium** parvum sporozoite surface proteins immunoprecipitated with monoclonal MAb 10C6. Lane 3 shows radiolabelled **Cryptosporidium** parvum sporozoite surface proteins immunoprecipitated with polyclonal anti-GP900.

DETD . . . confirms that polyclonal anti-GP900 antibody is an appropriate antibody for GP900 localization experiments and for detection of clones in a **Cryptosporidium** expression library.

DETD In order to determine whether GP900 similarly was shed from the surface of **Cryptosporidium** sporozoites, living sporozoites were allowed to glide on poly-L-lysine coated slides. Results are shown in FIG. 3.

DETD . . . the sporozoites with formaldehyde. MAb 7B3 was used because it was previously shown to detect only GP900 on immunoblots of **Cryptosporidium** sporozoite proteins. FIG. 3A shows that GP900 is present around the living sporozoite and is shed (FIG. 3B) from the. . .

DETD In order to show that GP900 is a glycoprotein, N-linked carbohydrate was enzymatically removed from **Cryptosporidium** parvum **oocyst**/sporozoite proteins and the remaining protein was as separated by **SDS**-PAGE and detected with MAb 10C6 by an immunoblot. Results are seen in FIG. 4.

DETD An anti-GP900 polyclonal antibody, affinity purified from **oocyst** /sporozoite antibodies on the protein expressed by a .lambda.gt11 clone S34 using S34 recombinant eluted antibody (S34 REA), also detected a. . .

DETD . . . of the second antibody, used to localize GP900 polyclonal antibodies, are concentrated. GP 900 was also seen in sporozoites within **oocysts** (data not shown). The rhoptries and dense granules were not labeled. No surface labeling of sporozoites and merozoites was observed.. . .

DETD The GP900 gene of **Cryptosporidium** parvum was isolated from a naturally infected neonatal calf (NINC) isolate. DNA from calves was used to prepare a .lambda.gt11. . .

DETD FIG. 6 shows that the DB8 probe of the NINC **Cryptosporidium** isolate hybridizes to a single DNA fragment in EcoRI, Bgl II and Hind III digests of the Iowa strand, indicating. . .

DETD The presence of abundant cysteines on a surface protein of **Cryptosporidium** which is functionally homologous to the circumsporozoite protein of malaria strongly suggests that these cysteines participate in binding phenomena and. . .

DETD . . . proteins were prepared according to Example 6. These various antibody preparations were used to probe an immunoblot of proteins from **Cryptosporidium** parvum **oocysts**/sporozoites. Results are shown in FIG. 7.

DETD FIG. 7 is an immunoblot of proteins obtained from **Cryptosporidium** parvum **oocysts** or sporozoites. Marker size in kDa is indicated. Lane 1 is the S34 antigen probed with pre-immune rabbit serum. Lanes. . .

DETD . . . shows that the pre-immune serum from the rabbit which received the S34 antigen is mildly reactive to two proteins of **Cryptosporidium** parvum. After immunization with the S34 antigen (lanes 2-4), the antisera react with a whole variety of proteins including GP900,. . .

DETD In order to determine whether native or recombinantly produced antibodies in fact inhibit **Cryptosporidium** infection and would, therefore, be viable reagents for provoking active or providing

passive immunity, or be useful for therapeutic purposes,. . .

DETD For these studies, MDCK cell monolayers were infected with **Cryptosporidium** parvum **oocysts** of the Iowa isolate in the presence of control reagents or immune sera, and colostrum was directed against a series of **Cryptosporidium** parvum **oocyst** antigens. Antisera and HBC Ig/sham HBC Ig were diluted 1:40 in cell culture media, such as RPMI, except for affinity. . .

DETD FIG. 8 is a graph showing **Cryptosporidium** parvum invasion into MDCK cells which were not treated (RPMI control=Bar 1) or were treated with fetal calf serum (bar. . .

DETD . . . calf serum (2), or sham hyperimmune bovine colostrum (SHAM-HBC) (3), collected from cows immunized with herd vaccines but not with **Cryptosporidium**. Recombinant S-34-glutathione-s-transferase fusion protein (S34-GST) at 100 nM and 1 .mu.M preincubated with 1:40 anti-S34-galactosidase (bar 4 shows 1 .mu.M). . .

DETD . . . absence of antibodies in controls or the presence of sham HBC anti-.beta.-galactosidases and anti-GP900 antibodies did not provide protection against **Cryptosporidium** infectivity, invasion or adhesion. On the other hand, antibodies raised against S34 (bar 7) and against Ag4 (bar 8 and bar 9), whether affinity purified (bar 9) or not (bar 8), provided good protection against **Cryptosporidium** infection. Affinity purified polyclonal antibody (bar 9) was the most active in this system and almost equal in activity to. . .

DETD . . . 50 (bar 6), 100 (bar 7) and 500 (bar 8) .mu.g/ml, was determined and compared to controls represented by untreated **oocysts** (bar 1), S34-.beta.-galactosidase antibodies (bar 2), HBC Ig (bar 3) and anti-**oocyst** antibodies (bar 4). Response is expressed in parasite-to-host nuclei ratio. Results are seen in FIG. 9.

DETD . . . 50% protection while 100 .mu.g/ml and 500 .mu.g/ml provided excellent to almost complete protection against invasion and intracellular development of **Cryptosporidium** sporozoites. These results further demonstrate the specific nature of the antibody/antigen reaction.

DETD In order to determine whether the native or recombinant antibodies raised against **Cryptosporidium** antigen GP900 or a fraction thereof are able to inhibit **Cryptosporidium** infection in vivo, the anti-S34-.beta.-galactosidase and anti-S19-.beta.-galactosidase antibodies were tested in a neonatal mice model. Results are seen in FIG.. . .

DETD FIG. 10 shows the in vivo effect of antibodies to GP900 recombinants on shedding of **oocysts** by neonatal mice infected with **Cryptosporidium**.

DETD FIG. 10 is a graph representing the amount of excretion of **Cryptosporidium oocysts** per day in mice treated with phosphate buffered saline (bar 1); anti-.beta.-galactosidase (bar 2); anti-Ag4-.beta.-galactosidase (bar 3); anti-S34-.beta.-galactosidase (bar 4);. . . 1:5 HBC Ig 40529 (bar 5); and paromomycin (bar 6). As seen in FIG. 10, anti-S34 (bar 4) reduced the **oocysts** shed by about 500 relative to control PBS (bar 1) and anti-.beta.-galactosidase antibody (bar 2). Although crude antisera was used,. . . 40529 (bar 5), the positive control antibody which had previously been shown to prevent cryptosporidial disease in calves challenged with **Cryptosporidium** (Infect. Immun., 61:4079-4084 (1993)).

DETD From the results obtained in these experiments, it is clear that clone S34 encodes a **Cryptosporidium** antigen and that the antibodies specifically raised against this antigen are able to inhibit the **Cryptosporidium** infection in vivo.

DETD . . . .beta.-galactosidase, Ag4-.beta.-galactosidase and S34-.beta.-galactosidase, as described in Example 13. In this assay, the same magnitude of inhibition of adhesion of **Cryptosporidium** sporozoites to CaCO-2 cells (mean O.D. 50% of control in ELISA) with a 1:50 dilution of anti S34-.beta.-galactosidase was conferred. . .

DETD In addition, these results were comparable to those seen when a 1:100 dilution of anti-**Cryptosporidium** murine ascites (48% inhibition) a polyclonal rabbit anti-**Cryptosporidium** antiserum (inhibition 51%) were previously assayed in this system (data not

shown). Similarly to the in vivo model, in this. . . Additionally, these results show that antibodies to recombinant GP900 correlate significantly with the inhibitory activity of HBC Ig 40529 and anti-**Cryptosporidium** antibodies from mouse and rabbit sources.

DETD . . . genomic DNA sequences depicted by SEQ ID NO: 1. Antibodies against the recombinant S34 protein are able to significantly inhibit **Cryptosporidium** infection in vitro and in vivo.

DETD Consequently, the results described above indicate the usefullness of the anti-S34 antibody for both anti-**Cryptosporidium** prophylaxis and therapy of a human or animal host.

DETD A. Identification of the **Cryptosporidium** Antigen designated P68

DETD A **Cryptosporidium** antigen designated P68 is an apical protein of sporozoites and merozoites. The protein has a size of between about 50-100. . .

DETD . . . (1992)). A recombinant eluted antibody from the clone identified a dominant 68 kDa protein on Western blot (FIG. 11) of **oocyst** sporozoite proteins and was localized to the anterior end of the sporozoite by indirect fluorescent antibody analysis.

DETD FIG. 11 is an immunoblot of AUCP isolate **oocyst**/sporozoite proteins. Lane 1 was detected with polyclonal anti-sporozoite/**oocyst** antibodies which had been affinity purified on the S19 fusion protein (S19-REA). As seen in FIG. 11, an immunoblot with. . .

DETD . . . 3), and anti-GST fusion protein (bar 4) and (bar 5) antibodies raised against native GST were not effective in inhibiting **Cryptosporidium** infection. The two anti-GST/S19 (bar 6) and (bar 7) antibodies raised against a recombinant fusion protein of S19-glutathione-s-transferase clone inhibited the **Cryptosporidium** invasion by 46% and 33% relative to control. Both were more inhibitory than an anti-**oocyst**/sporozoite (bar 8) antibody made in rabbits. The anti-GST antibodies, pre-immune antibodies and 5% fetal calf serum (FCS), did not inhibit. . .

DETD FIG. 13 is a graph showing the effect of anti-S19 antibody on **oocyst** excretion in vivo in a CD neonatal mouse model as described in Example 14. FIG. 13 shows that antibodies to S19-GST raised in two rabbits (bar 3) and (bar 4) significantly decreased **oocyst** excretion as compared to animals treated with anti-GST antibody (bar 2) or without treatment (bar 1).

DETD In order to evaluate the immune response to **Cryptosporidium** infection in HIV infected or in healthy individuals, mucosal and systemic lymphoproliferative responses were studied using recombinant GP900 stimulation of mucosal lymphocytes in the gastrointestinal mucosa of a rhesus macaque infected with SIV and **Cryptosporidium** which did not have the clinical disease. This model was established to determine what type of mucosal responses are correlated. . .

DETD . . . are described in Example 15. Briefly, a rhesus macaque mucosal immunity model was developed to evaluate the immune response to **Cryptosporidium** in normal and in HIV infected individuals. A mucosal lymphocyte proliferation experiment, results of which are shown and described in. . . at necropsy from the duodenum/jejunum, ileum, and colon of a clinically well, (no weight loss, no diarrhea) SIV-infected macaque, experimentally **Cryptosporidium** infected rhesus with a low CD4 count of 250/mm.sup.3. The animal intermittently excreted **oocysts**. The experiment was designed to look at T cell stimulation and used concanavalin A (con A) as a T cell. . .

DETD . . . S34-GST (S34) or the control peptide GST, indicating that there was no systemic proliferative response in the face of known **Cryptosporidium** infection without disease.

DETD . . . immune responses to these antigens in the face of SIV infection in a clinically healthy animal with intermittent shedding of **Cryptosporidium**.

DETD A second rhesus macaque that was SIV and **Cryptosporidium** infected remained clinically well but developed diarrhea and was excreting **Cryptosporidium** as her blood CD4 count fell below 100/mm.sup.3. Lymphoproliferative responses to sporozoite antigens, S34-GST, S19-GST, GST and concanavalin A, could. . .

DETD These results, when taken together with the observations from the first animal, suggest that **Cryptosporidium** infection may cause chronic diarrhea when the mucosal proliferative response to cryptosporidial antigens ceases, with a fall in production of the antibody in the lamina propria to specific **Cryptosporidium** antigens to subprotective levels.

DETD III. In vitro Inhibition of **Cryptosporidium** parvum Infection

DETD The in vitro inhibition of the invasion and intracellular development of **Cryptosporidium** described in Example 11 for GP900 and in Example 18 for P68 protein was shown to occur as a function of anti-**Cryptosporidium** titer. This was evidenced by its correlation with the corresponding immunoglobulin concentration in protective colostrum (HBC), and by the lack. . . of SHAM colostrum (SHAM-HBC). In a supportive experiment described in Example 13, HBC Ig was also shown to significantly inhibit **Cryptosporidium** parvum adhesion in the CaCO-2 cell line, thus providing a potential mechanism for inhibition of invasion and infection.

DETD The in vitro ability of HBC to prevent **Cryptosporidium** infectivity was shown to be mediated only by specific anti-**Cryptosporidium** antibodies eluted from **Cryptosporidium** . Elution from fetal calf serum and SHAM-colostrum did not produce inhibition of the infection.

DETD The studies conducted in support of this invention show that antibodies to specific **Cryptosporidium** antigens are also responsible for the in vitro effect of the HBC Ig fraction. Additionally, the inhibition in the in. . .

DETD IV. In vivo Inhibition of **Cryptosporidium** parvum Infection

DETD Studies of the inhibitory effect of the polyclonal antibody of the invention on the **Cryptosporidium** parvum infection in vivo were performed according to the procedure described in Example 17.

DETD . . . vitro inhibition studies. Assessment of in vivo efficacy of HBC was performed in newborn, colostrum deprived, Holstein calves challenged with **oocysts** of **Cryptosporidium** parvum. The efficacy of the immune colostrum preparation for protecting the treated calves from **Cryptosporidium** parvum infection was demonstrated in statistically significant differences between treated and control animals in cumulative fecal scores ($p<0.01$ by one. . .

DETD . . . occurred in the treated group whereas all of the calves in the control group showed some signs of dehydration. The **oocyst** output was dramatically reduced in the treated group (<10.sup.3 **oocysts** per total fecal output, the limit of detection) when compared to the control group (geometric mean **oocyst** output=5.62.times.10.sup.8).

DETD These results clearly show that the immune colostrum treatment effectively reduced the initial colonization by **Cryptosporidium** parvum parasites and suppressed the intestinal proliferation of the **Cryptosporidium** parvum parasites which were not initially neutralized.

DETD . . . S34 is an effective antigen for the production of passive and active immune products for immunization, prophylaxis and treatment of **Cryptosporidium** infections.

DETD . . . to native or recombinant protein or a glycoprotein of the invention are useful for treatment by providing a protection against **Cryptosporidium** infections.

DETD Anti- **Cryptosporidium** parvum polyclonal antibodies recognizing the cloned polypeptides are preferred over monoclonal antibodies because they recognize multiple epitopes on the target. . .

DETD VIII. Biologically Derived or Recombinant Anti- **Cryptosporidium** Vaccines

DETD A recombinant vaccine is produced by identifying the relevant antigen or antigens of **Cryptosporidium**, cloning them and expressing them using suitable vectors. This approach yields immunogens which are reproducible in sufficiently large quantities to allow preparation of a vaccine for active immunization. Recombinant vaccines are useful for immunization of the potential **Cryptosporidium** host, such as for inoculation of cows, to produce the host's own antibodies against a

Cryptosporidium infection. Additionally, the recombinant vaccines may be used for production of passive immunotherapeutic agents. For example, when the cow is. . .

DETD These vaccines are also useful for widespread use in calves to provide primary protection against **Cryptosporidium** infection. Providing the herd with anti-**Cryptosporidium** immunity decreases the risk for waterborne outbreaks of cryptosporidiosis in areas where the watershed includes dairy lands. This provides a. . .

DETD The anti-**Cryptosporidium** vaccine of the invention contains a **Cryptosporidium** antigen identified by the invention, modified in such a way that it is incapable of producing the **Cryptosporidium** symptoms but is capable of eliciting the production of specific protective antibodies against the disease when introduced in the body. A DNA or RNA vaccine for prevention and treatment of infections caused by protozoan **Cryptosporidium** species (**Cryptosporidium**) in humans and other mammals was developed by utilizing newly identified and isolated DNA (SEQ ID NOs: 1-4) and amino acid (SEQ ID NOs: 5 and 6) sequences of the **Cryptosporidium** pathogen designated GP900.

DETD A hybrid vector comprising a DNA segment that encodes the protein antigen able to bind selectively and specifically to anti-**Cryptosporidium** antibodies operatively coupled to the vector was prepared and expressed. This includes preparation of recombinant vaccines using the viral expression. . .

DETD . . . or RNA vaccines or native immunity are produced according to the methods described Ibid. Briefly, DNA vectors encoding the deactivated anti-**Cryptosporidium** antigen DNA or RNA are injected, preferably intramuscularly, wherein said antigen is produced and elicits its own immune responses in the form of a specific anti-**Cryptosporidium** antigen antibody thereby providing its own immunity and/or cell mediated responses.

DETD The current invention provides means for suitable immunoprotection against **Cryptosporidium** infections or for a therapeutic use of immune agents produced according to the invention.

DETD A method for immunotherapeutic treatment, retardation, or inhibition of **Cryptosporidium** infection comprises administering to a subject in need of such treatment an amount of an anti-**Cryptosporidium** polyclonal or monoclonal antibody prepared according to the invention, effective to provide immunity against the invasion of **Cryptosporidium** or effective to inhibit the existing **Cryptosporidium** infection.

DETD A method of prophylaxis of **Cryptosporidium** infection comprises administering to a subject in need of such treatment a vaccine comprising the protein or recombinant protein of this invention capable of endogenous development of an inhibitory amount of anti-**Cryptosporidium** parvum antibodies.

DETD For passive immunotherapy **Cryptosporidium** infected hosts, the polypeptide is first combined with appropriate adjuvants and used for the immunization of cows or other donor. . .

DETD In AIDS patients **Cryptosporidium** parvum may cause a devastating disease for which there is no treatment. Understanding of the organism and the pathophysiology of the disease it produces, and development of treatment, are very important steps in the treatment of **Cryptosporidium** infection.

DETD Therefore, the protective activity of proteins of the invention was studied in an in vitro model of **Cryptosporidium** sporozoite invasion and intracellular development in Madin-Darby Canine Kidney (MDCK) cells. These studies validated the studies performed in the model. . .

DETD GP900 and P68 proteins are therefore the first **Cryptosporidium** vaccine candidates for preparation of active vaccines and passive immune products for human and animal use in combatting **Cryptosporidium** infections.

DETD Active vaccines and passive immune products prepared from the protein of the invention are suitable for prevention and therapy of **Cryptosporidium** parvum infections in AIDS or other patients suffering from cryptosporidiosis.

DETD . . . effective. One of the therapeutic approaches for treatment of chronic cryptosporidiosis according to the invention is the use of hyperimmune anti-**Cryptosporidium** antibodies prepared against the protein antigen of the invention which may be given orally to humans to provide a therapeutic. . .

DETD **Cryptosporidium** parvum Parasites

DETD This example illustrates the protocol used for isolation of **Cryptosporidium** parvum parasites.

DETD **Oocysts** of the Iowa and AUCP-1 isolates of **Cryptosporidium** parvum were passaged through neonatal calves at the Animal Resources Services, University of California, Davis or obtained from a commercial source (Pat Mason) and the **oocysts** were purified and excysted. The detailed protocol is described in Infect. Immun., 61:4079 (1993).

DETD Preparation of Murine Anti-**Oocyst** and Anti-Sporozoite Polyclonal and Monoclonal Antibodies

DETD This example describes the procedure used for preparation of murine anti-**oocyst** and anti-sporozoite polyclonal and monoclonal antibodies.

DETD 10 week-old female BALB/c mice were immunized four times intraperitoneally with approximately 5.times.10.sup.5 sonicated **Cryptosporidium** parvum **oocysts**. The polyclonal antibody fraction of the ascites which was shown to react with the **Cryptosporidium** parvum sporozoite surface, the **oocyst** surface, and/or with internal antigens of the **oocysts**, was assessed by an IFA as described in Infect. Immun., 60(12):5132 (1992).

DETD For monoclonal antibody production, mice were immunized intravenously with the supernatant from sonicated **Cryptosporidium** parvum **oocysts** three days before fusion as described in J. Immunol., 123:1548 (1979) and J. Parasitol., 68:1029 (1982). Hybridoma supernatants were used. . .

DETD Anti-**Cryptosporidium** parvum Antibodies Eluted from Western Blot

DETD For **SDS**-PAGE, 2.times.10.sup.9 **oocysts** were lysed by 5 cycles of freeze-thawing in 1% Triton Buffer (150 mM Na Cl; 100 mM EDTA; and 1%. . . protease inhibitors: 100 .mu.M E64, 100 .mu.M chymotrypsin, 100 .mu.M pepstatin, and 100 .mu.M leupeptin; and 1.6 mM PMSF, and **boiled** in sample buffer (SB). Proteins were electrophoresed in 5-15% gradient gels described in Nature, 227:680 (1971) and blotted onto nitrocellulose. . .

DETD The Triton X-100 (1%) soluble fraction of 2.times.10.sup.8 **oocysts** was immunoprecipitated with MAb 10C6. A>900 kD MW species was identified in gels stained with Coomassie blue in water and excised. Frozen gel containing 2.times.10.sup.7 **oocyst** /sporozoites was pulverized and emulsified in 150 PI of PBS and 150 .mu.l complete Freund's adjuvant (CFA) for intraperitoneal (IP) immunization. . .

DETD **Oocysts** (10.sup.6 lane) were solubilized in 5% .beta.ME (.beta.-mercaptoethanol) containing a sample buffer, resolved by **SDS**-PAGE and subjected to immunoblotting. Proteins were visualized after incubation with primary antibody with .sup.125 I-labeled Protein A followed by autoradiography. . .

DETD To identify the molecular targets of protective antibody, total **Cryptosporidium** parvum sporozoite and sporozoite/**oocyst** proteins were **boiled** in sample buffer (SB), resolved in 5-15% gradient gels by **SDS**-PAGE and Western blotted with HBC Ig. In addition, sporozoite/**oocyst** proteins solubilized in Triton-X 100 were immunoprecipitated with HBC Ig at dilutions 1/1,000; 1/5,000; 1/10,000; 1/50,000 and 1/100,000. **Cryptosporidium** parvum proteins immunoprecipitated under the same conditions but with SHAM-HBC Ig at dilutions 1/1,000 to 1/10,000 were used as controls. Immunoprecipitates were also resolved by **SDS**-PAGE and Western blotted. Western blots of HBC Ig immunoprecipitates were developed with HBC Ig (dilution 1:1,000) and SHAM immunoprecipitates were. . .

DETD DNA was purified from 1.times.10.sup.9 **Cryptosporidium** parvum **oocysts** as described in Example 1. DNA was digested with the

restriction enzymes according to procedures provided by the manufacturer Promega.. . .

DETD Surface Radioiodination and Immunoprecipitation of **Cryptosporidium** Sporozoite Proteins

DETD This example describes the methods used for surface radio-iodination and immunoprecipitation of **Cryptosporidium** sporozoite proteins.

DETD **Oocysts** were bleached, excysted and separated from sporozoites prior to iodination of the sporozoite surface and immunoprecipitation of surface proteins as. . .

DETD . . . 0.5% Triton X-100, pH 7.4) at 100,000.times.g for 1 hour at 40.degree. C. An aliquot of membrane proteins in 2% **SDS** 5% p-sample buffer was prepared for total sporozoite surface protein analysis. Aliquots of membrane proteins extracted in 2% **SDS** were diluted with 9 volumes NETT plus 1% high quality bovine serum albumin (BSA) obtained from Sigma; 1 volume 1%. . . overnight incubation. Protein A Sepharose 4B beads were added to immobilize the immunoprecipitated proteins. Parasite proteins were solubilized in 2% **SDS** sample buffer containing .beta.-mercaptoethanol. Samples were **boiled** 5 minutes and separated by 5-15% gradient **SDS**-PAGE.

DETD Immunoelectronmicroscopic Localization of GP900 in **Cryptosporidium** parvum-Infected Rat Intestinal Tissue

DETD This example describes the immunoelectronmicroscopic methods used for localization of GP900 antigen in **Cryptosporidium** parvum infected rat intestinal tissue.

DETD **Oocysts** were used to inoculate confluent Madin Darby Canine Kidney (MDCK) cell monolayers for in vitro inhibition assays of sporozoite invasion. . . chamber slides from Nunc Inc., Napersville, Ill. containing 10.sup.5 MDCK cells were overlaid with 400 1 RPMI medium containing 1.5.times.10.sup.5 **oocysts** and antibody or colostrum samples to be tested for inhibitory capacity. Each experimental data point was an average of the. . .

DETD Controls included hyperimmune bovine colostrum 40529 Ig (HBC Ig) raised against **Cryptosporidium oocysts** and sporozoites and SHAM-HBC raised against a herd vaccine at ImmuCell Corp, Portland, Me.

DETD . . . in Example 11. The antibody, at concentrations of 10, 50, 100 and 500 .mu.g/ml in RPMI, was incubated with excysted **oocysts** on MDCK cell monolayers for two hours. The wells were washed out and refilled with RPMI.

DETD Control wells contained equal amounts of **oocysts** and RPMI alone, S34-GST at 100 nM, anti-**oocyst**/sporozoite antibody at a 1:40 dilution and HBC Ig 40529 at a 1:40 dilution. As described in FIG. 9 invasion/intracellular development. . .

DETD Inhibition of **Cryptosporidium** Invasion and Intracellular Development in MDCK cells with MAb 10C6

DETD This example describes studies performed to detect inhibition of **Cryptosporidium** invasion and intracellular development in vitro using monoclonal antibodies.

DETD **Cryptosporidium oocysts** of the AUCP-1 isolate were excysted and three sporozoite monoclonal antibodies, MAb 10C6, 7B3 and E6, were prepared as described. . .

DETD . . . and intracellular development were found to be inhibited by >95% compared to the control antibody. Sequential observation of viable, unfixed **Cryptosporidium** sporozoites by differential phase contrast microscopy after addition of MAb 10C6 revealed initial reactivity of the MAb with the surface. . .

DETD In order to determine whether GP900 was shed by the **Cryptosporidium** sporozoite in the absence of a specific antibody, living sporozoites were allowed to glide on poly-L-lysine coated microscopic slides. Slides. . .

DETD The two anti-GST/S19 antibodies inhibited the **Cryptosporidium** invasion by 46% and 33% relative to control. Both were more inhibitory than an anti-**oocyst**/sporozoite antibody made in rabbits. The anti-GST antibodies did not inhibit invasion and intracellular development.

DETD In Vivo Inhibition of **Cryptosporidium** Infection in Mice

Challenged with **Cryptosporidium Oocysts** with Anti-S34-.beta.-Galactosidase and Anti-S19-.beta.-Galactosidase Polyclonal Antibodies

DETD This example describes the method used for determination of the in vivo inhibition of **Cryptosporidium** infection of mice challenged with **Cryptosporidium oocysts** and treated with specific anti-S34-.beta.-galactosidase (FIG. 10) and anti-S19-.beta.-galactosidase (FIG. 13) polyclonal antibodies.

DETD Anti-.beta.-galactosidase,anti-S34-.beta.-galactosidase,anti-Ag4-.beta.-galactosidase and HBC Ig 40529 were tested for inhibitory activity in a neonatal mouse model of **Cryptosporidium** infection.

DETD Three experiments were performed and the data pooled. In each experiment 5 neonatal mice per group were infected with **Cryptosporidium** and were fed either 20 .mu.l control PBS, 20 .mu.l of the 3 rabbit antibodies or 20 .mu.l of a. . . human AIDS patients for cryptosporidiosis, was given to mice in 2 experiments. Infection was scored as the mean number of **oocysts** shed per day during a 5 day collection period.

DETD . . . challenge protection experiment in which antibodies were assayed in vivo in groups of 7 CD1 neonatal mice challenged with 10.sup.4 **oocysts** orally on day 6. **Oocyst** output was scored in Sheather's solution and is expressed as 10.sup.5/ ml. Antisera were diluted 1:2 in 50 mM NaHCO.sub.3.. . . 2 is anti-GST antibodies, 3 is anti-S19-GST antibodies from rabbit #1, 4 is anti-S19 antibodies from rabbit #2. Inhibition of **oocyst** excretion relative to control was greater than 45% for the antisera from both rabbits immunized with S19-GST when compared to preimmune sera or antisera to GST alone. When 10 fold more **oocysts** were used in the challenge the inhibitory effect decreased suggesting that the S19 antibodies titrated a particular molecular event.

DETD Effect of Specific **Cryptosporidium** Antigens Mucosal on Lymphocyte Proliferation in Rhesus Macaques Infected with SIV and **Cryptosporidium** parvum

DETD . . . for detection of lymphocyte proliferation specific for S34 and S19 in the mucosa of rhesus macaques infected with SIV and **Cryptosporidium** parvum.

DETD The animals were challenged with whole viable **Cryptosporidium oocysts** and their clinical state and blood CD4 count were monitored for protracted periods (up to 14 months) before they were sacrificed for evaluation of mucosal lymphocyte responses to **Cryptosporidium** antigens. The animal's entire GI tract was removed and divided into duodenum/jejunum, ileum and colon segments. The tissues were dissected. . .

DETD The proteins of the invention bind to antibodies which specifically bind to epitopes of **Cryptosporidium** parvum. These **Cryptosporidium** parvum epitopes are also recognized by B and T cells. The proteins mentioned above are produced in large amounts by reinserting the **Cryptosporidium** parvum DNA from the different clones described in Section I, above, into an expression vector such as pGEX, pET-9d, or. . .

DETD . . . from E. coli. Following protein expression, the vector sequences are easily eliminated so that the subsequent immunogenic protein contains only **Cryptosporidium** sequences. These expression systems are commercially available and their use is standard in the art.

DETD Peptides, polypeptides, glycopeptides or proteins comprising epitopes of **Cryptosporidium** parvum recognized by B and/or T cells are produced in large amounts by recloning, as described in Example 19, above.. . .

DETD Preparation of Anti-**Cryptosporidium** parvum Vaccine

DETD This example illustrates procedure for anti-**Cryptosporidium** parvum vaccine.

DETD Vaccine use of recombinant **Cryptosporidium** antigens.

DETD

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<211> LENGTH: 5318
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- <400> SEQUENCE: 15
- Met Gly Ser Lys Val Tyr Ile Pro Tyr. . .
CLM What is claimed is:
1. An isolated and sequenced **Cryptosporidium** antigen GP900 detected at the surface of **Cryptosporidium** sporozoites or merozoites comprising 1721 amino acid sequence SEQ ID NO: 5, wherein said antigen has a molecular weight greater. . . amino acid sequence SEQ ID NO: 5, and wherein said anti-GP900 antibodies structurally interact with the GP900 antigen and inhibit **Cryptosporidium** infection, invasion or adhesion.

. . . The antigen of claim 5 detected with the monoclonal anti-GP900 antibodies obtained by animal immunization with a supernatant from sonicated **Cryptosporidium** parvum ocysts.

8. An isolated and sequenced **Cryptosporidium** antigen P68 which is an apical protein of **Cryptosporidium** sporozoites or merozoites comprising 503 amino acid sequence SEQ ID NO: 6, wherein said antigen has a molecular weight between. . . amino acid sequence SEQ ID NO: 6; and wherein said anti-P68 antibodies structurally interact with the P68 antigen and inhibit **Cryptosporidium** sporozoites or merozoites infection, invasion or adhesion.

12. A purified recombinant **Cryptosporidium** GP900 antigen protein comprising 1721 amino acid sequence SEQ ID NO: 5, or a variant or mutant thereof; wherein said. . .

L4 ANSWER 10 OF 14 USPATFULL
ACCESSION NUMBER: 1999:78543 USPATFULL
TITLE: Method for nucleic acid isolation using supercritical fluids
INVENTOR(S): Nivens, David E., 11912 Kingsgate Rd., Knoxville, TN, United States 37911
Applegate, Bruce M., 3700 Sutherland Ave. #Q2, Knoxville, TN, United States 37911

	NUMBER	KIND	DATE	
PATENT INFORMATION:	US 5922536		19990713	
APPLICATION INFO.:	US 1996-733816		19961018	(8)

DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Housel, James C.
ASSISTANT EXAMINER: Ryan, V.
LEGAL REPRESENTATIVE: Knobbe, Martens, Olson & Bear, LLP
NUMBER OF CLAIMS: 11
EXEMPLARY CLAIM: 1

NUMBER OF DRAWINGS: 4 Drawing Figure(s); 4 Drawing Page(s)
LINE COUNT: 817
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

SUMM . . . or combinations of these, followed by dissolution of the cell membrane with alkali and detergents such as sodium dodecyl sulfate (**SDS**) (Maniatis et al., 1989; Tsai et al., Appl. Environ. Microbiol., 57:1070-1074, 1991; Bej et al., Appl. Environ. Microbiol., 57:1013-1017, 1991).. . .

DETD . . . lysate rather than removing the lysate from the lytic agent. The supercritical properties of CO.sub.2 thus eliminate the need for **SDS** as a lytic reagent. These current art procedures not only result in loss of sample, but in some cases are. . .

DETD . . . methods allowing a more rapid detection of microorganisms contained in water samples, including waterborne pathogens such as E. coli, Shigella, **Cryptosporidium** and Giardia. The apparatus can be used to detect microorganisms present in recreational waters, source water and potable water.

DETD . . . Mycobacterium and Rhodococcus. Results from lysis of these species under supercritical conditions were compared to those obtained using a conventional **SDS** lysis procedure (Table 1). For Pseudomonas, Sphingomonas and E. coli, the lytic action of supercritical fluids is comparable to that of **SDS** and, in the case of Rhodococcus and Mycobacterium, supercritical fluids are a better lytic agent. Dilution plate counts of lysed bacteria showed no growth after treatment with **SDS** or exposure to supercritical conditions, indicating that both methods result in loss of bacterial viability.

DETD TABLE 1

	% SFF Lysis by AODC count	Standard Deviation	% **SDS** Lysis by AODC count	Standard Deviation
E. coli	88.83	2.98	90.23	1.85
Pseudomonas	90.31	6.88	94.09	8.39
Sphingomonas				

. . .

DETD . . . this point on, the procedure was the same for both sets of controls. Cells were extracted with 30 .mu.l 10% **SDS**, 3 .mu.l Proteinase K (20 mg/ml) (Sigma, St. Louis, Mo.). The samples were incubated for 1 hour at 37.degree. C.,. . .

DETD . . . T4 kinase. Sample DNA aliquots and 16s DNA standards were prepared in 0.4 M NaOH (final volume 0.5 ml) and **boiled** for 10 min. Samples and standards were blotted onto Biotrans TM nylon membrane (ICN, Irvine, Calif.) using a slot blot apparatus. . . 80.degree. C. for 1 hour. The blot was prehybridized in 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 7% **SDS** for 1 hour at 37.degree. C. The .sup.32 P-labeled probe was added to the blot and incubated overnight. The blot. . . four times using a high stringency wash buffer (20 mM Tris-HCl, pH 7-8, 10 mM NaCl, 1 mM EDTA, 0.5% **SDS**, 37.degree. C.). The blot was dried, exposed to x-ray film and hybridization signals were quantitated by densitometry using a Visage. . .

DETD . . . exposure times (100.degree. C., 400 atm, 30 min) produced the greatest yield of DNA compared to that obtained by standard **SDS** lysis procedures. Genomic DNA appears to be completely unaffected by the extremes of temperature and pressure used in supercritical fluid. . .

DETD . . . recovery to 45%, while chloroform:methanol (1:1) pretreatment resulted in recovery of 78% of the DNA compared to that of the **SDS**-treated bacterial cultures. The differing recoveries may be due to differences in cell membranes compared to the other bacterial species (i.e.,. . .

DETD Samples of **Cryptosporidium** muris **oocysts** were prepared by filtration onto GF/F glass fiber filters. Each filter contained approximately 2.63.times.10.sup.7 **oocysts**. The filters were exposed to supercritical conditions of 400 atm at 100.degree. C. for 30 minutes and the nucleic acids. . .

L4 ANSWER 11 OF 14 USPATFULL
ACCESSION NUMBER: 1999:40580 USPATFULL
TITLE: Recombinant neospora antigens and their uses
INVENTOR(S): Conrad, Patricia A., Davis, CA, United States
Barr, Bradd C., Davis, CA, United States
Anderson, Mark L., Davis, CA, United States
Sverlow, Karen W., Vacaville, CA, United States
Louie, Kitland, Davis, CA, United States
PATENT ASSIGNEE(S): The Regents of the University of California, Oakland, CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 5889166		19990330
APPLICATION INFO.:	US 1996-645951		19960510 (8)

DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Caputa, Anthony C.
ASSISTANT EXAMINER: Navarro, Mark
LEGAL REPRESENTATIVE: Townsend and Townsend and Crew LLP
NUMBER OF CLAIMS: 4
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 6 Drawing Figure(s); 3 Drawing Page(s)
LINE COUNT: 1991
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

SUMM . . . matched probe. Typically stringent conditions for a Southern blot protocol involve washing at room temperature with a 5.times. SSC, 0.1% **SDS** wash.

DETD . . . to the sequences for this gene in other coccidial parasites. Alignment of these sequences with published sequences of Neospora caninum, **Cryptosporidium** parvum, Sarcocystis muris and Toxoplasma gondii showed that the bovine Neospora isolate is genotypically unique.

DETD . . . biological samples. These sequences can be used to detect all stages of the Neospora life cycle (e.g., tachyzoites, bradyzoites, and **oocysts**) in biological samples from both the bovine host and the definitive host. A variety of methods of specific DNA and. . .

DETD . . . are particularly useful for the diagnosis of neosporosis and identification of the source of Neospora parasite stages (tachyzoites, bradyzoites and **oocysts**) in various animal hosts.

DETD . . . Neospora. The compositions of the invention can also be used to treat the definitive host to prevent the shedding of **oocysts** and subsequent transfer to cattle. The compositions for administration to either cattle or the definitive host can comprise tachyzoite and/or. . .

DETD . . . 4 different antisera to T. gondii (Tg1-4). Antiserum Tg1 was produced by the infection of a rabbit with live sporulated **oocysts** of the ME-49 strain (Lindsay & Dubey, supra) of T. gondii and used at a 1:400 dilution. Toxoplasma gondii antiserum. . .

DETD . . . antigen expression of the different parasites and variations in the methods used to produce the antisera (i.e. immunization with cysts, **oocysts** or tachyzoite lysates). For example, tissue cyst wall antigens that reacted with antiserum to H. hammondi appeared to be lacking. . .

DETD . . . DNA was prepared as follows. Briefly, the parasite or control cell pellets were suspended in 1.0 ml STE with 0.5% **SDS** treated with proteinase K (100 .mu.g/ml) and RNAase (100 .mu.g/ml) then extracted twice with phenol, once with phenol-chloroform-isoamyl alcohol, and. . .

DETD . . . After hybridization, the membranes were washed twice for 5 min each at room temperature in 5.times. SSC and 0.1% (w/v) **SDS**, and then washed twice for 5 min each at room temperature in 0.5.times. SSC and 0.1% (w/v) **SDS**. Membrane blocking, antibody incubations, signal generation and detection were performed as described by the manufacturer. Membranes were exposed to Kodak. . .

DETD . . . with the probes overnight at 42.degree. C. The blots were washed at 42.degree. C. (2.times.20 min.) in 6M Urea, 0.4% **SDS**, 0.5.times. SSC and rinsed with 20.times. SSC prior to autoradiography.

DETD . . . when the Eco R1 insert from clone N54 was used as a probe, the wash protocol was adjusted to 0.4% **SDS**, 0.5.times. SSC at 45.degree. C. (2.times.20 min.).

DETD . . . Both recombinant antigens and parasite antigens were quantitated with the BCA Protein Assay (Pierce), denatured in Laemmli's sample buffer and **boiled** for 5 minutes prior to electrophoresis. **SDS**-PAGE and Western blots were performed under standard conditions.

L4 ANSWER 12 OF 14 USPATFULL

ACCESSION NUMBER: 97:56538 USPATFULL

TITLE: **Cryptosporidium** hybrid vector and transformed host cells

INVENTOR(S): Petersen, Carolyn, Berkeley, CA, United States
Leech, James, Daly City, CA, United States
Nelson, Richard C., San Francisco, CA, United States
Gut, Jiri, Novato, CA, United States

PATENT ASSIGNEE(S): The Regents of the University of California, Oakland, CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 5643772		19970701
APPLICATION INFO.:	US 1995-415751		19950403 (8)

RELATED APPLN. INFO.: Continuation of Ser. No. US 1993-71880, filed on 1 Jun 1993, now abandoned which is a continuation-in-part of Ser. No. US 1992-891301, filed on 29 May 1992, now abandoned

DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Housel, James C.
ASSISTANT EXAMINER: Portner, Ginny Allen
LEGAL REPRESENTATIVE: Verny, Hana
NUMBER OF CLAIMS: 4
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 7 Drawing Figure(s); 4 Drawing Page(s)
LINE COUNT: 2279
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

TI **Cryptosporidium** hybrid vector and transformed host cells

AB The invention comprises a **Cryptosporidium** hybrid vector comprising a regulatory DNA segment operably coupled to a DNA fragment encoding a polypeptide to which anti-**Cryptosporidium** antibodies specifically bind and transformed host cells comprising the hybrid vectors.

SUMM . . . relates to a novel polypeptide comprising an amino acid sequence capable of specifically binding antibodies raised against the protozoan pathogen **Cryptosporidium** spp. (**Cryptosporidium**). This invention also relates to DNA and RNA segments which encode such polypeptides. These polypeptides bind antibodies that afford protection. . . . or RNA sequences encoding them, and they may be used for the immunization of humans or animals against infection by **Cryptosporidium**. These polypeptides may also be used for the production of antibodies suitable to counter **Cryptosporidium** infection. Finally, this invention relates to the use of these polypeptides and antibodies raised against them in diagnosing infection by **Cryptosporidium** and diagnostic kits.

SUMM **Cryptosporidium** are parasitic agents causing infection in a wide variety of animals including birds, reptiles and mammals. C. parvum is believed. . . . chemotherapy is available at the present time to counter this disease. Moreover, the understanding of the biology and biochemistry of **Cryptosporidium**, as well as the pathophysiology of cryptosporidiosis is still at an early stage.

SUMM The C. parvum infection is initiated by the ingestion of **oocysts**

, the excystation of **oocysts** with release of sporozoites and the invasion of gut epithelial cells by sporozoites. Thereafter, the intracellular forms mature and release. . . sexual cycle. The sexual cycle of C. parvum also occurs in the gut and results in the production of sporulated **oocysts**, some of which may excyst before being shed. In persistent infection of an immunocompromised host, both the merozoite and the. . .

SUMM . . . oral transfer of anti-C. parvum hyperimmune bovine colostral immunoglobulin (HBC Ig). HBC Ig has been shown to react with numerous **oocyst** and sporozoite proteins on Western blots and to be therapeutic in neonatal mice. Whole hyperimmune bovine colostrum (HBC) has also. . .

SUMM . . . exposed on the surface of C. parvum. These antigens are examples of targets of monoclonal antibodies raised against the corresponding **oocysts**/sporozoites. These monoclonal antibodies have been shown to prevent or attenuate infection in studies using animals challenged with C. parvum. Monoclonal. . .

SUMM . . . invention relates to a biologically pure polypeptide comprising a biologically pure, isolated peptide capable of selectively and specifically binding to anti-**Cryptosporidium** antibodies.

SUMM This invention also relates to a method of retarding, inhibiting, or countering **Cryptosporidium** infection of a subject's cells comprising administering to a subject in need of such treatment an amount of an anti-**Cryptosporidium** antibody effective to retard the invasion by and/or development of **Cryptosporidium** of the subject's cells.

SUMM In addition, this invention comprises a method of diagnosing **Cryptosporidium** infection of a subject, comprising

SUMM contacting a body substance obtained from the subject with an anti-**Cryptosporidium** antibody; and

SUMM detecting any selective binding of the antibody to any antigenic **Cryptosporidium** peptide present in the body substance.

SUMM This invention, in addition to the above, also encompasses a method of diagnosing **Cryptosporidium** infection of a subject, comprising

SUMM detecting any selective binding of the polypeptide to any anti-**Cryptosporidium** antibodies in the body fluid.

SUMM Also part of this invention is a **Cryptosporidium** diagnostic kit, comprising

SUMM anti-**Cryptosporidium** specific antibodies; and

SUMM Furthermore, this invention also provides a **Cryptosporidium** infection diagnostic kit, comprising

DRWD FIG. 1 depicts the inhibition of **Cryptosporidium** infection of epithelial cells by HBC Ig. 100-1000 .mu.g/ml IgG resulted in a significant reduction ($p<0.01$) in the mean number. . .

DRWD FIG. 3 depicts the results of another specific anti-**Cryptosporidium** antibody assay. HBC Ig antibodies (50-100 .mu.g/ml IgG) eluted from C. parvum Western blots were utilized in a MDCK in vitro assay. The experimental data were normalized to SHAM-HBC IgG controls (75 .mu.g/ml IgG). Significant inhibition of **Cryptosporidium** infectivity was observed for cultures treated with anti-**Cryptosporidium** Ab eluted from Western blot (Eluted Ab) and HBC Ig (100 .mu.g/ml IgG) (HBC Ig) with respect to SHAM-HBC Ig. . .

DRWD FIG. 4 shows a Western Blot of C. parvum proteins developed with HBC Ig or SHAM-HBC Ig. **Oocyst**/sporozoite proteins were either blotted (lane 1) or immunoprecipitated with HBC Ig at dilutions 1/1,000 (lane 2), 1/5,000 (lane 3), and. . .

DRWD . . . an aliquot of the membrane proteins was directly resuspended in SB and electrophoresed (lane 1). Alternatively, radiolabeled sporozoite membranes were **SDS** solubilized (lane 2) or Triton X-100 solubilized (lane 3) prior to immunoprecipitation with affinity bound HBC Ig. Proteins were resolved by **SDS**-PAGE, and autoradiographed for 8 hrs.

DETD . . . symptoms produced by infection of the parasite. The polypeptides of this invention are also useful for the early detection of **Cryptosporidium** infection. The inventors targeted the

protein components of the parasite for their work. Protein extracts of C. parvum were screened. . . such polypeptides, viral DNA expression libraries were constructed and then screened with polyclonal anti-C. parvum antibodies reactive with sporozoites and **oocysts** of the parasite. A large number of colonies were screened. The number of clones was significantly reduced when only those. . .

DETD . . . the present peptides may be attained by contacting the peptide for each clone provided herein with polyclonal antibodies raised against **Cryptosporidium** or with monoclonal antibodies obtained by the fusion of, e.g., a myeloma cell line with an anti-**Cryptosporidium** antibody producing lymphocyte. The binding of the peptide in the biologically pure peptide to the antibodies may be determined by. . .

DETD . . . combinations and repeats thereof, and complementary and degenerate sequences thereof encoding the polypeptide(s). The DNA segments and fragments of different **Cryptosporidium** clone groups are shown in the tables provided with the examples. Also provided in the tables are the deduced amino. . .

DETD Also an important part of this invention is a method of diagnosing **Cryptosporidium** infection, that comprises

DETD contacting a body substance with an anti-**Cryptosporidium** antibody having specificity for the polypeptide of this invention; and

DETD detecting any selective binding of the antibody to any antigenic **Cryptosporidium** peptides present in the body substance. The detection of the antibody-polypeptide complex may be conducted by any method known in. . .

DETD Also provided herein is a method of diagnosing **Cryptosporidium** infection, that comprises

DETD detecting any selective binding of the polypeptide to any anti-**Cryptosporidium** antibodies in the body substance. As in the previous case, the present antibody-polypeptide binding complex may be detected by a. . .

DETD . . . a newborn calf model of acute crysptosporidiosis, HBC was shown to protect the animal from Crysptosporidium infection, to reduce the **oocysts** output to below the limit of detection, and to produce substantially no dehydration in the HBC treated animals. Moreover, an. . .

DETD . . . antibodies or components, as shown by the inhibitory effect evidenced by the total antibodies eluted from a Western-blot of Crysptosporidium sporozoite/**oocyst** proteins. Fayer et al reported in 1990 that the antibodies in HBC were responsible for the protective activity of HBC. . .

DETD Thus, also part of this invention is a method of retarding, inhibiting or countering **Cryptosporidium** infection of a subject's cells comprising administering to a subject in need of such treatment an amount of the polypeptide of this invention capable of eliciting from the subject a host cell invasion and/or development inhibitory amount of anti-**Cryptosporidium** antibodies. Several **Cryptosporidium** polypeptides of this invention have been shown to elicit antibodies which inhibit **Cryptosporidium** infectivity and/or development inside the cell. In one preferred embodiment, the polypeptide of the invention, suitable for eliciting anti-**Cryptosporidium** antibodies in a mammal, comprises the S7, S24, S19, S34 or S2 polypeptides and other polypeptides encompassed by the corresponding. . .

DETD This invention also provides a method of retarding, inhibiting or countering a **Cryptosporidium** infection of a subject's cells comprising administering to a subject in need of such treatment an amount of an antibody capable of binding to one or more of the polypeptides described above effective to retard the **Cryptosporidium** invasion of and/or development in the subject's cells. Antibodies selectively binding to several **Cryptosporidium** polypeptides have been shown by the inventors and others to inhibit **Cryptosporidium** infectivity in vitro. In one preferred embodiment, the antibody comprises anti-S7, anti-S24, anti-S19, anti-S34, or anti-S2 antibodies or mixtures thereof,. . . the

anti-S19, anti-S34 and anti-S2 antibodies and other polypeptides encompassed by the corresponding entire proteins. Typically, the antibodies specific for **Cryptosporidium** may be administered in an amount of about 0.01 to 100 g, and more preferably about 1 to 35 g.. . .

DETD Still part of this invention is a kit for the diagnosis of **Cryptosporidium** infection, that comprises

DETD . . . with cryptosporidiosis. Even at the early stages where the parasite is commencing invasion of a subject's cells, some amount of **Cryptosporidium** specific antibody may be detected in serum.

DETD Also provided herein is another **Cryptosporidium** diagnostic kit, that comprises

DETD anti-**Cryptosporidium** antibodies having specificity for one of the polypeptides of this invention; and

DETD Thus, kit may be utilized for the detection of **Cryptosporidium** polypeptides, a sign that there is parasite present in the subject being tested.

DETD **Cryptosporidium** sp. **oocysts** isolated from patients with AIDS at San Francisco General Hospital were used in the production of polyclonal and monoclonal antibodies. . .

DETD **Oocysts** were isolated by resuspension of 1 vol. of feces with 2 vol. of a saturated NaCl solution. All subsequent procedures. . . done at 4.degree. C. After centrifugation at 1,000 g, the supernatant was recovered and the procedure repeated 3 times. The **oocysts** were recovered from the pooled supernatants by centrifugation, purified further in a 55%-27.5%-14% sucrose gradient at 1000 g for 20 min., and stored in PBS. Prior to use, the **oocysts** were sterilized by incubation in 15% commercial bleach, and washed by repeated centrifugation and re-suspension in PBS. The purified **oocysts** were excysted by incubation in Rpmi medium (gibco) with the addition of 0.75% sodium taurocholate (Sigma), pH 7, for 40-60. . .

DETD Sporozoites were separated from unexcysted **oocysts** and debris by filtration through a polycarbonate 3 .mu.m pore size membrane (Millipore).

DETD **Cryptosporidium oocysts** from calves (Dr. Bruce Anderson, University of Idaho) were used for the isolation of DNA for the construction of the lambda gt11 genomic expression libraries. **Cryptosporidium** parvum **oocysts** of an AUCP-1 isolate (Dr. Byron Blagburn, Auburn University, Auburn, Ala.) propagated in Holstein calves were used for Western blots. . .

DETD Preparation of Murine Polyclonal and Anti-**oocyst**/Sporozoite MAbs

DETD 10 week-old female BALB/c mice were immunized four times intraperitoneally with approximately 5.times.10.sup.5 sonicated C. parvum **oocysts**. The ascites were extracted and the antibodies isolated there- from. The polyclonal antibody fraction of the ascites was shown to react with the C. parvum sporozoite surface, the **oocyst** surface and internal antigens of the **oocysts** as assessed by an IFA as described in Petersen et al., Infect. Immun. 60(12):5132 (1992).

DETD For monoclonal antibody production, mice were immunized intravenously with the supernatant from sonicated C. parvum **oocysts** three days before fusion as previously described by Kearney et al and Danforth et al (Kearney et al., J. Immunol.. . .

DETD A C. parvum extract was prepared from a lysate using 2% **SDS** and 1% Triton X-100, and immunoprecipitated as described by Leech et al (Leech et al., J. Exp. Med. 159:1567(1984)). Monoclonal . . . immune complexes collected with protein A/G agarose beads. The C. parvum polypeptides from the immune complexes were separated on 5% **SDS** -PAGE gels and analyzed using a Western blot.

DETD . . . the membrane pellet and the soluble fraction containing cytoplasmic proteins were collected. An aliquot of the membrane pellet was directly **boiled** in SB, and stored at -70.degree. C. (sporozoite membrane proteins).

DETD The remaining membrane material was divided into two radiolabeled samples. Prior to immunoprecipitation, one aliquot was extracted by

boiling in SB as above, followed by addition of 9 vol. of NETT (0.15M NaCl; 5 mM EDTA; 0.5M Tris; 0.5% Triton X-100; pH 7.4) with 1% BSA (Sigma), 1% Triton-X 100, and protease inhibitors (**SDS** solubilized membranes). The other radiolabeled membrane sample was extracted directly with 3 vol. of NETT with 1% BSA, 1% Triton. . .

DETD The immunoprecipitates were washed sequentially with NETT buffer alone and NETT containing 1% BSA (Sigma) or 500 mM NaCl, then **boiled** in SB and stored at -70.degree. C. Proteins were separated in 5-15% gradient gels by **SDS**-PAGE, and processed for autoradiography using X-Omat film (Kodak). Iodination controls consisted of tri-chloroacetic acid (TCA) precipitates of the soluble fraction. . .

DETD . . . Corporation (Portland, Me.) and was prepared by repeated parenteral immunization of Holstein cows during pre-parturition with partially excysted C. parvum **oocysts**. Immunogens were emulsified in Freund's adjuvant.

DETD . . . mg/ml IgG) of the same lot was used in the in vitro inhibition of development studies of Example 34 below. Anti-**Cryptosporidium** Ab titers were determined independently several times for each colostrum preparation. HBC Ig (lot #40529) had an average anti-**Cryptosporidium** antibody titer of 1/176,000 U/ml for a 43 .mu.g/ .mu.l IgG concentration by ELISA. SHAM-HBC Ig (lot #41038) had an approximately ten fold lower average antibody titer to **Cryptosporidium** antigens by ELISA (17,000 U/ml for a 45 .mu.g/ .mu.l IgG concentration), probably due to natural infection of the animals. . .

DETD . . . (control group) and all other parameters were equal. All 8 animals were challenged at 12 hrs. of age with 5.times.10.sup.6 **oocysts** of C. parvum. All animals were fed 100 ml of SHAM-HBC or HBC every 24 hrs. and 2 qt milk. . .

DETD . . . samples were taken every 12 hrs. Fecal and dehydration scores were tabulated from days 5-7, the days of peak patency. **Oocyst** shedding was tabulated over days 5-9 post infection in 3/4 treated animals and 2/4 controls. Samples from the remaining animals were not available. **Oocyst** shedding was measured by mixing 1 vol. fecal sample with 4 vol. Sheather's solution and enumerating the refractive **oocysts** in a hemocytometer. Confirmation of the **oocyst** counts was performed with a commercial immunofluorescence kit utilizing a monoclonal anti-**oocyst** antibody (Merifluor, Meridian Diagnostics, Cincinnati, Ohio).

DETD . . . occurred in the treated group whereas all of the calves in the control group showed some signs of dehydration. The **oocyst** output was dramatically reduced in the treated group (<10.sup.3 **oocysts** per total fecal output, the limit of detection) when compared to the control group (geometric mean **oocyst** output=5.62.times.10.sup.8).

DETD The two libraries were screened with polyclonal anti-C. parvum **oocyst**/sporozoite antibodies obtained as described in Example 2 and positive clones were plaque purified as described by Petersen et al., Infect.. . .

DETD . . . from IPTG-induced confluent plaque lifts of purified lambda gt11 clones were selected by affinity chromatography from a polyclonal anti-C. parvum **oocyst**/sporozoite antibody preparation on the respective plaquelifts. The antibodies were then eluted with 10 mM glycine pH 2.6, and 150 mM. . .

DETD Indirect IFA Localization of Endogenous Antigens in Sporozoites and **Oocysts**

DETD Slides containing air-dried, acetone-fixed sporozoites and **oocysts** were incubated with antibodies isolated as described in Example 8 above for 1 hr in a humidified chamber, washed with. . .

DETD . . . of the S24 group on the other hand, reacted diffusely with the fixed sporozoite on IFA but not with the **oocyst**.

DETD One hundred million **oocysts** were suspended in 500 .mu.l of a protease inhibitor cocktail containing 100 .mu.M leupeptin (Sigma), 100 .mu.M chymostatin (Sigma), 100. . .

DETD . . . from clones of the S34 group bound to a polypeptide of apparent molecular weight >900 kD on Western blots of **oocyst**/sporozoite

proteins. This polypeptide migrated faster than titin at 2500 kD and slower than nebulin at 900 kD.

DETD Western blots probed with the murine **oocyst**/sporozoite antibodies from which the eluted antibodies were isolated indicated that the antibodies reacted with many different **oocyst**/sporozoite antigens. Control eluted antibodies prepared from wild type lambda gt11 clones which only express beta-galactosidase showed substantially no binding to **oocyst**/sporozoite polypeptides. This clearly indicates that the elution process yielded highly specific antibody. It is unclear if the fact that multiple. . .

DETD A whole **oocyst**/sporozoite lysate was electrophoresed in a 5% **SDS**-PAGE gel and Western blotted with HBC IgG. At least 10 polypeptides were identified. Because of the compression effect of the. . .

DETD Immunoprecipitation of Triton X-100 soluble **oocyst**/sporozoite proteins with monoclonal antibody 10C6 followed by **SDS**-PAGE and Western blot with HBC Ig confirmed that the antigenic sites reside on the same molecule. Control lanes indicate that additional antigens are heavy and light chains from antibodies in the fractionated immune complexes. Coomassie blue staining of **SDS**-PAGE gels suggested that the >900 kD antigen predominates over other proteins in **oocyst**/sporozoite lysates.

DETD Immunoprecipitation of Triton X-100 extracted **oocyst** /sporozoite polypeptides with monoclonal antibody 10C6 followed by Western blot with monoclonal antibodies 10C6, 7B3 and E6 evidenced that all three. . .

DETD The 7B3 monoclonal antibody also identified a 38 kD apparent molecular weight antigen on a Western blot of whole **oocyst**/sporozoite lysates but not on Western blots of whole sporozoite lysates. This suggests that a molecule from **oocysts** that is insoluble in Triton-X-100 also reacts with monoclonal antibody 7B3, the monoclonal antibody reacting with the anterior portion of. . .

DETD Sporozoites were obtained from **oocysts** isolated from calves infected with the AUCP-1 strain of C. parvum. The sporozoites were cultured in vitro on Madin-Darby canine. . .

DETD A Western blot analysis of **oocyst**/sporozoite polypeptides immunoprecipitated with monoclonal antibody 10C6 indicated that the S34 eluted antibodies bind to the >900 kD polypeptide that is. . .

DETD . . . 37.degree. C. with N-glycosidase F (Boehringer Mannhelm, EC 3.2.2.18) according to Boehringer Mannhelm instructions, and then electrophoresed in a 5% **SDS** PAGE gel and Western blotted. To control proteolysis during the incubation, the C. parvum lysate was incubated under the same. . .

DETD The **oocyst**/sporozoite proteins were treated with N glycosidase F, and then electrophoresed on a 5% **SDS** PAGE gel and Western blotted with the 10C6, 7B3 and E6 monoclonal antibodies. None of the monoclonal antibodies detected an. . .

DETD 4) Immunoprecipitation of Triton X-100 soluble **oocyst** /sporozoite polypeptides and **SDS** extraction of the Triton X-100 insoluble pellet indicate the >900 kD apparent molecular weight glycoprotein to be mostly Triton X-100. . .

DETD . . . clone S34 was determined by chain termination DNA sequencing (Sanger, F., et. al., Proc. Nat. Acad. Sci. (USA) 74:5463 (1977)). **Cryptosporidium** DNA inserts were excised from lambda gt11 with EcoRI and ligated into m13 (Messing, J., Methods in Enzymology 101:20 (1983)). . .

DETD . . . E. coli. The vector sequences may be easily eliminated following protein expression so that the subsequent immunogenic protein contains only **Cryptosporidium** sequences. These expression systems are commercially available and their use is standard in the art.

DETD When used to passively immunize **Cryptosporidium** infected animals, the polypeptide is first combined with appropriate adjuvants and used for the immunization of cows or other donor. . .

DETD These antibodies are used to detect **Cryptosporidium** antigens in body substances, for example, stools of populations at risk of cryptosporidial infection by, e,g., collecting stool samples (Manual. .

DETD For SDS-PAGE, 2.times.10.sup.9 **oocysts** were lysed by 5 cycles of freeze-thawing in 1% Triton Buffer (150 mM Na CI; 100 mM EDTA; and 1%. . . Triton X-100), in the presence of protease inhibitors (100 .mu.M E64, chymotrypsin, pepstatin, and leupeptin; and 1.6 mM PMSF), and boiled in Sample Buffer (SB). Proteins were electrophoresed in 5-15% gradient gels (Laemmli, U. K., "Cleavage of Structural Proteins During the. . . (1971)) and blotted onto nitrocellulose at 0.7 amp. for 8 hrs. (Petersen, C., et al., "Characterization of an Mr>900,000 **Cryptosporidium** parvum Sporozoites Glycoprotein Recognized by Hyperimmune Bovine Colostral Immunoglobulin", Inf. & Immun. 60(12):5132 (1992)). Western blots were incubated with HBC. . . for 3 min., followed by addition of a 1/10 volume of 2M Tris buffer, pH 8 (Tilley, M., et al., " **Cryptosporidium** parvum (Apicomplexa: Cryptosporidiidae) **Oocyst** and Sporozoite Antigens Recognized by Bovine Colostral Antibodies", Inf. Imm. 58:2966 (1990)). Eluted antibodies were filter sterilized and concentrated to. . .

DETD . . . follows to quantify the effect of antibody on the infection of epithelial cells by C. parvum (Gut J., et al., "**Cryptosporidium** parvum: In vitro Cultivation in Madin-Darby Canine Kidney Cells" J. Protozool. 386:72 (1991)). MDCK cells were maintained in RPMI-1640 with. . .

DETD The cells were then rinsed in RPMI without FCS for 30 minutes, exposed to 2.times.10.sup.6 purified **oocysts** resuspended in RPMI medium containing HBC Ig (lot#40529) (1000; 500; 200; 100; and 50 .mu.g/ml IgG), SHAM-HBC Ig (lot #41038). . . 5% FCS (300-500 .mu.g/ml IgG). In some experiments, controls were also conducted consisting of MDCK cells infected with C. parvum **oocysts** resuspended in RPMI medium with the addition of glycine buffer at the same concentration utilized for the cultures treated with. . .

DETD . . . cultures were incubated for 2 hrs. at 37.degree. C., rinsed 4 times with RPMI to remove extracellular sporozoites and unexcysted **oocysts**, and reincubated for an additional 21 hrs. period in the presence of the respective antibody reagents described above. Monolayers were. . .

DETD To identify the molecular targets of protective antibody, total C. parvum sporozoite and sporozoite/**oocyst** proteins were **boiled** in sample buffer (SB), resolved in 5-15% gradient gels by **SDS**-PAGE and Western blotted with HBC Ig. In addition, sporozoite/**oocyst** proteins solubilized in Triton-X 100 were immunoprecipitated with HBC Ig at dilutions 1/1,000; 1/5,000; 1/10,000; 1/50,000 and 1/100,000. Controls were. . . proteins immunoprecipitated under the same conditions but with SHAM-HBC Ig at dilutions 1/1,000 to 1/10,000. Immunoprecipitates were also resolved by **SDS**-PAGE and Western blotted. Western blots of HBC Ig immunoprecipitates were developed with HBC Ig (dil 1/1,000) and SHAM immunoprecipitates were. . .

DETD The in vitro assay described in Example 26 above was used to determine inhibition of **Cryptosporidium** invasion and/or intracellular development as a function of HBC Ig titer. HBC Ig, at concentrations ranging from 100-1,000 .mu.g/ml IgG,. . . was found not to significantly inhibit C. parvum infectivity relative to 5% FCS controls, although this reagent does contain some anti-**Cryptosporidium** activity by ELISA as shown in FIG. 2. In addition, HBC Ig (100-1,000 .mu.g/ml IgG) significantly inhibited **Cryptosporidium** invasion and/or development when compared to SHAM-HBC Ig controls by 45-55%. Specific anti-C. parvum antibodies were affinity purified from Western blot (about 50-100 .mu.g IgG/ml). This eluted antibody also inhibited **Cryptosporidium** invasion/development relative to SHAM-HBC Ig controls, and controls with the addition of glycine buffer or FCS as shown in FIG.. . .

DETD When total **oocyst**/sporozoite proteins were immunoprecipitated with HBC Ig at different concentrations and blotted, two sporozoite molecules of >900 kD and about 250. . . parvum antigenic proteins recognized by HBC Ig are expressed by sporozoites as evidenced by the

comparison of total sporozoite and **oocyst**/sporozoite proteins recognized by HBC Ig in Western blots (data not shown). **Cryptosporidium** sporozoites were also radioiodinated to identify antigens localized to their surface. Twenty-two surface iodinatable sporozoite proteins were resolved by **SDS**-PAGE (FIG. 5, lane 1). Protective anti-**Cryptosporidium** antibodies (lot#140529) immunoprecipitated most of these surface labeled sporozoite proteins. The number of radiolabeled immunoprecipitated proteins resolved by gel electrophoresis differed for membranes solubilized with **SDS** or with Triton X-100. Nineteen labeled sporozoite surface proteins extracted with **SDS** were specifically immunoprecipitated by HBC Ig (FIG. 5, lane 2) including a >900 kD and about 250 kD molecules (FIG.. . .

DETD . . . Freund's Complete Adjuvant. The mice receiving the S34 antigen were boosted with 5 to 10 .mu.g fusion protein excised from **SDS** polyacrylamide gel, minced and extruded through a hypodermic needle, which was administered i.p. After 7 total immunizations, the production of. . . induced by the i.p. inoculation of animals with Freund's Complete Adjuvant. The resulting ascites were used to probe western blotted **Cryptosporidium** parvum **oocyst** lysates. As expected, the anti-S2 ascites recognized about 45 kD band, the anti-S19 ascites recognized the about 68 kD band,. . .

DETD In Vitro Inhibition of C. parvum Infectivity by anti-S19, S34, and S2 **Cryptosporidium** Protein Antibodies

DETD . . . utilized to quantify the effect of antibody on the infection of epithelial cells by C. parvum (Doyle, P., et al., "Anti-**Cryptosporidium** parvum Antibodies Inhibit Infectivity In Vitro and In Vivo", Inf. Immun. (1993), in press). Briefly, MDCK cells were maintained in. . .

DETD The cells were then rinsed in RPMI medium without FCS for 30 min., exposed to 2.times.10.sup.6 purified **oocysts** resuspended in RPMI medium containing the antibodies, i.e., anti-S19 ascites; anti-S34 ascites; anti-S2 ascites; hyperimmune bovine colostrum immunoglobulin (HBC Ig);. . .

DETD . . . cultures were incubated for 2 hrs. at 37.degree. C., rinsed 4 times with RPMI to remove extracellular sporozoites and unexcysted **oocysts**, and reincubated for an additional 21 hrs. period in the presence of the respective antibody reagents as described above. Monolayers. . .

DETD The ascites were tested for their ability to neutralize **Cryptosporidium** sporozoite infectivity using a modification of the **Cryptosporidium** invasion assay described by Tilley et al (Tilley et al., Infec. Immun. 59:1002-1007 1991). Briefly, MDBK cells were seeded into. . . Eagle medium (DME)/2% Fetal Bovine Serum (FBS) and grown for 3 days to greater than 90% confluency. Purified C. parvum **oocysts** from a virulent calf isolate, recently passaged in a newborn calf, were excysted by exposure to 0.75% (w/v) sodium taurocholate. . .

DETD . . . for 15 min. at room temperature and permeabilized with methanol for 10 min. After washing the monolayer with PBS, Bovine anti-**Cryptosporidium** IgG was added at a dilution of about 1 mg/ml IgG in PBS/1% Normal Goat Serum (NGS) for I hr.. . .

DETD . . . NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 176 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GlnHisPheLeuLeuGlnLeuGluProGlnAspAsnGlnGlnLeuLeu
151015
GlnLeuGluValGlnAlaAsnGlnLeuLeuLeuProLeuSerLysAla
202530

ThrThrThrThrThrThrLeuAsnProIleIleThrThrThrThrGln
354045
LysProThrThrThrThrThrLysValProGlyLysProProIle
505560
AlaThrThrThrThrThrLeuLysProIleValThrThrThrThrThr
65707580
LysAlaThrThrThrThrThrThrValProThrThrThrThrThr
859095
ThrLysArgAspGluMetThrThrThrThrThrProLeuProAspIle
100105110
GlyAspIleGluIleThrProIleProIleGluLysMetLeuAspLys
115120125
TyrThrArgMetIleTyrAspTyrAsnSerGlyLeuLeuLeuAspSer
130135140
AsnAspGluProIleProGlySerGlnAlaGlyGlnIleAlaAspThr
145150155160
SerAsnLeuPheProGlySerAsnSerGlnGluTyrTrpPheThrAsn
165170175
(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 201 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
SerIleGluMetSerThrLeuValArgLysLeuAlaProAsnPheThr
151015
AlaGluAlaValMetAlaAspGlySerPheLysLysValSerLeuSer
202530
AspTyrArgGlyLysTyrValValLeuPhePheTyrProLeuAsnPhe
354045
ThrPheValCysProSerGluIleLeuAlaPheAsnGlnAlaGlnLys
505560
AspPheGluLysLeuGlyValGlnLeuLeuSerCysAlaGlnLeuIle
65707580
LeuAsnThrProMetLeuHisGlyAspValLeuLeuLeuAsnLysVal
859095
GluLeuAspGlnSerIleSerHisLeuSerLeuThrHisLeuIleGln
100105110
LeuAlaArgThrMetValTyrPheLeuGluGluGluGlyIleAlaLeu
115120125
ArgGlyLeuPheIleIleAspLysGluGlyArgValValArgSerGlu
130135140
ValIleTyrAspLeuProLeuGlyArgSerValGluGluThrLeuArg
145150155160
ValIleAspAlaLeuGlnPheThrGluThrTyrGlyGluValCysPro
165170175
AlaAsnTrpLysLysGlyGlnLysGlyMetSerAlaThrHisGluGly
180185190
ValSerSerTyrLeuLysAspSerPhe
195200
(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 361 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
GluPheProAspArgSerLeuAspPheThrIleProProValAlaGly
151015
HisAsnSerCysSerIleIleValGlyValSerGlyAspGlyLysIle
202530
HisValSerProTyrGlySerLysAspValSerLeuIleSerAlaPro
354045
IleGlnProSerGluLeuPheAsnGluValTyrCysAspThrCysThr
505560
AlaLysTyrGlyAlaIleHisSerGlyTyrGlnThrSerAlaAspPhe
65707580
ValThrThrLeuProThrThrThrGlyAlaAlaGlyGlnProThrThr
859095
ThrThrThrGlySerProSerLysProThrThrThrThrThrIleXaa
100105110
GlyAsnAsnAsnHisAsnAsnSerXaaSerAsnHisTyrAsnAsnAsn
115120125
SerLysThrAsnAsnAsnAsnAsnLysGlySerArgXaaAlaThr
130135140
AsnSerHisAsnAsnAsnAsnIleLysAlaAsnSerTyrAsnAsnAsn
145150155160
AsnLysSerAsnAsnAsnAsnAsnAsnSerAlaAsnAspAsnTyr
165170175
TyrTyrGlnGluArgArgAsnAspAsnAsnAsnAspThrIleThrXaa
180185190
TyrArgXaaHisXaaAsnTyrThrAsnProAsnXaaLysAspValGly
195200205
XaaValHisLysAsnAspLeuXaaLeuXaaGlnTrpPheIleIleArg
210215220
LeuXaaXaaXaaThrAsnSerArgPheSerSerArgThrAsnSerXaa
225230235240
TyrLysGlnPheIleProArgPheLysLeuThrArgValLeuValTyr
245250255
GlnLeuIleGlnTrpLeuValPheHisLeuIleGlnAsnGlnValIle
260265270
XaaTyrIleHisIleProIleLysGlnCysLeuValTyrArgTyrHis
275280285
IleLeuLeuLeuArgIleXaaGlnLeuIleLeuMetLysLeuArgPhe
290295300
ThrAsnXaaTyrThrHisTrpLeuProIleGlySerSerGlnPheAsp
305310315320
SerValGlnSerArgAsnTrpXaaIleValCysProIleSerAspGlu
325330335
IleMetAsnGlyThrIleAlaGlyIleValSerGlyIleSerAlaSer
340345350
GluSerLeuLeuSerGlnLysSerLeu
355360
(2) INFORMATION FOR SEQ. . . NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 361 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are
identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
ArgValProArgXaaLysPheGlyPheHisAsnSerSerSerSerTrp
51015
ProXaaGlnLeuPheAsnAsnSerTrpCysGluArgArgTrpLysAsn
202530
SerArgLysProIleArgPheXaaGlyCysLeuSerAsnLysCysSer
354045

AsnThrThrPheXaaValIleGlnXaaSerLeuLeuArgHisLeuTyr
505560
CysGluValTrpCysAsnSerLeuTrpIleSerAsnPheSerXaaPhe
65707580
ArgAsnAsnThrSerTyrTyrAsnTrpSerArgArgThrThrAsnAsn
859095
TyrTyrAsnTrpLysSerLysGlnThrAsnTyrTyrTyrHisTyrLeu
100105110
ArgGlnGlnGlnProGlnGlnLeuLeuIleGlnSerLeuGlnGlnGln
115120125
LeuLysAsnGlnGlnGlnGlnGlnGlnArgPheGlnValSerHis
130135140
GlnXaaProGlnGlnGlnGlnHisXaaSerGlnXaaLeuGlnGlnGln
145150155160
GlnGlnLysGlnGlnGlnGlnGlnGlnGlnCysGlnArgGlnLeu
165170175
LeuLeuProArgGluThrLysXaaGlnGlnGlnArgHisHisTyrLeu
180185190
IleSerValThrLeuLysLeuHisGlnSerGlnLeuLysArgCysTrp
195200205
IleSerThrGlnGluXaaPheMetThrIleThrValValTyrTyrXaa
210215220
ThrLeuMetMetAsnGlnPheGlnValLeuLysGlnAspLysXaaLeu
225230235240
IleGlnAlaIleTyrSerGlnValGlnThrHisLysSerThrGlyLeu
245250255
ProIleAspProMetValGlyLeuProPheAspProLysSerGlyAsn
260265270
LeuValHisProTyrThrAsnGlnThrMetSerGlyLeuSerValSer
275280285
TyrLeuAlaAlaLysAsnLeuThrValAspThrAspGluThrThrVal
290295300
TyrGlnLeuIleHisSerLeuValThrHisTrpIleGlnSerValXaa
305310315320
PheArgSerIleGlnLysLeuValAsnCysLeuSerAsnIleArgXaa
325330335
AspAsnGluTrpAsnAsnCysArgTyrCysPheArgAsnPheCysLys
340345350
XaaValIleIleIleSerGluIleAla
355360
(2) INFORMATION FOR SEQ. . . NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 362 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are
identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:
AlaSerSerGlnIleGluValTrpIleSerGlnPheLeuGlnXaaLeu
51015
AlaIleThrAlaValGlnXaaXaaLeuValXaaAlaAlaMetGluLys
202530
PheThrXaaAlaHisThrValLeuArgMetSerLeuXaaXaaValLeu
354045
GlnTyrAsnLeuLeuSerTyrSerMetLysPheIleAlaThrLeuVal
505560
LeuArgSerMetValGlnPheThrLeuAspIleLysLeuGlnLeuIle
65707580
SerXaaGlnHisPheLeuLeuGlnLeuGluProGlnAspAsnGlnGln
859095
LeuLeuGlnLeuGluValGlnAlaAsnGlnLeuLeuLeuProLeuSer

100105110
LysAlaThrThrThrThrThrLeuAsnProIleIleThrThrThr
115120125
ThrGlnLysProThrThrThrThrThrLysValProGlyLysPro
130135140
ProIleAlaThrThrThrThrLeuLysProIleValThrThrThr
145150155160
ThrThrLysAlaThrThrThrThrThrThrValProThrThrThr
165170175
ThrThrThrLysArgAspGluMetThrThrThrThrProLeuPro
180185190
AspIleGlyAspIleGluIleThrProIleProIleGluLysMetLeu
195200205
AspLysTyrThrArgMetIleTyrAspTyrAsnSerGlyLeuLeuLeu
210215220
AspSerAsnAspGluProIleProGlySerGlnAlaGlyGlnIleAla
225230235240
AspThrSerAsnLeuPheProGlySerAsnSerGlnGluTyrTrpPhe
245250255
ThrAsnXaaSerAsnGlyTrpSerSerIleXaaSerLysIleArgXaa
260265270
PheSerThrSerIleTyrGlnSerAsnAsnValTrpPheIleGlyIle
275280285
IleSerCysCysXaaGluPheAspSerXaaTyrXaaXaaAsnTyrGly
290295300
LeuProIleAspThrLeuThrGlyTyrProLeuAspProValSerLeu
305310315320
IleProPheAsnProGluThrGlyGluLeuPheValGlnTyrGlnMet
325330335
ArgXaaXaaMetGluGlnLeuGlnValLeuPheGlnGluPheLeuGln
340345350
ValSerHisTyrTyrLeuArgAsnArgSer
355360
(2) INFORMATION FOR SEQ. . . NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 362 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
GlyAlaIleSerGluIleIleMetThrHisLeuGlnLysPheLeuLys
151015
GlnTyrLeuGlnLeuPheHisSerLeuSerHisLeuIleLeuAspLys
202530
GlnPheThrSerPheTrpIleGluArgAsnGlnThrAspTrpIleGln
354045
TrpValThrSerGluCysIleAsnTrpXaaThrValValSerSerVal
505560
SerThrValLysPheLeuAlaAlaArgTyrAspThrAspLysProAsp
65707580
IleValXaaLeuValTyrGlyCysThrLysLeuProAspPheGlySer
859095
AsnGlyArgProThrIleGlySerIleGlyLysProValLeuLeuXaa
100105110
ValXaaThrTrpGluXaaIleAlaCysIleSerTyrLeuSerCysLeu
115120125
ArgThrTrpAsnTrpPheIleIleArgValXaaXaaXaaThrThrVal
130135140
IleValIleAsnHisSerCysValLeuIleGlnHisLeuPheAsnTrp
145150155160

AspTrpCysAsnPheAsnValThrAspIleArgXaaTrpCysArgCys
165170175
CysCysHisPheValSerLeuGlySerSerSerCysArgTrpHisCys
180185190
CysCysCysCysCysCysCysPheCysCysCysCysCysAsnTyrTrp
195200205
LeuXaaCysCysCysCysCysGlyTyrTrpTrpLeuThrTrpAsnLeu
210215220
CysCysCysCysCysCysTrpPheLeuSerCysCysCysAsnAspTrp
225230235240
IleLysSerCysCysGlyCysCysCysLeuArgXaaTrpXaaXaaXaa
245250255
LeuValCysLeuAspPheGlnLeuXaaXaaLeuLeuValValLeuArg
260265270
LeuGlnLeuXaaXaaGluValLeuLeuArgAsnGlnLeuLysPheAsp
275280285
IleGlnSerGluLeuHisHisThrSerGlnTyrLysCysArgAsnLys
290295300
LeuHisXaaIleThrGlnLysValValLeuGluHisLeuLeuGluArg
305310315320
HisProXaaAsnArgMetGlyLeuArgGluPhePheHisArgArgSer
325330335
HisGlnLeuLeuLeuAsnSerCysTyrGlyGlnLeuLeuGluGluLeu
340345350
XaaAsnProAsnPheTyrLeuGlyThrArg
355360
(2) INFORMATION FOR SEQ. . . NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 361 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
SerAspPheXaaAspAsnAsnAspSerLeuAlaGluIleProGluThr
151015
IleProAlaIleValProPheIleIleSerSerAspIleGlyGlnThr
202530
IleHisGlnPheLeuAspXaaThrGluSerAsnXaaLeuAspProMet
354045
GlyAsnGlnXaaValTyrGlnLeuValAsnArgSerPheIleSerIle
505560
AsnCysGlnIleLeuSerSerLysIleXaaTyrArgXaaThrArgHis
65707580
CysLeuIleGlyIleTrpMetTyrXaaIleThrXaaPheTrpIleLys
859095
TrpLysThrAsnHisTrpIleAsnTrpXaaThrSerThrLeuValSer
100105110
LeuAsnLeuGlyIleAsnCysLeuTyrGlnLeuPheValLeuLeuGlu
115120125
AsnLeuGluLeuValHisHisXaaSerLeuIleIleAsnHisCysTyr
130135140
SerHisLysSerPheLeuCysThrTyrProThrSerPheGlnLeuGly
145150155160
LeuValXaaPheGlnCysHisArgTyrGlnValMetValSerLeuLeu
165170175
LeuSerPheArgLeuSerTrpXaaXaaXaaLeuSerLeuAlaLeuLeu
180185190
LeuLeuLeuLeuLeuLeuLeuLeuLeuLeuLeuXaaLeuLeuAla
195200205
LeuMetLeuLeuLeuLeuTrpLeuLeuValAlaTyrLeuGluProLeu

210215220
LeuLeuLeuLeuLeuValPheGluLeuLeuXaaXaaLeuAsp
225230235240
XaaGluLeuLeuTrpLeuLeuLeuProXaaIleValValValValVal
245250255
GlyLeuLeuGlyLeuProValValValValValGlyCysProAlaAla
260265270
ProValValValGlySerValValThrLysSerAlaGluValXaaTyr
275280285
ProGluXaaIleAlaProTyrPheAlaValGlnValSerGlnXaaThr
290295300
SerLeuAsnAsnSerGluGlyCysIleGlyAlaLeuIleArgGluThr
305310315320
SerLeuGluProTyrGlyLeuThrXaaIlePheProSerProLeuThr
325330335
ProThrIleIleGluGlnLeuLeuTrpProAlaThrGlyGlyIleVal
340345350
LysSerLysLeuLeuSerGlyAsnSer
355360
(2) INFORMATION FOR SEQ. . . NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 361 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
GluArgPheLeuArgXaaXaaXaaLeuThrCysArgAsnSerXaaAsn
151015
AsnThrCysAsnCysSerIleHisTyrLeuIleXaaTyrTrpThrAsn
202530
AsnSerProValSerGlyLeuAsnGlyIleLysLeuThrGlySerAsn
354045
GlyXaaProValSerValSerIleGlyLysProXaaPheHisGlnTyr
505560
GlnLeuSerAsnSerXaaGlnGlnAspMetIleProIleAsnGlnThr
65707580
LeuPheAspTrpTyrMetAspValLeuAsnTyrLeuIleLeuAspGln
859095
MetGluAspGlnProLeuAspGlnLeuValAsnGlnTyrSerCysGlu
100105110
PheGluProGlyAsnLysLeuLeuValSerAlaIleCysProAlaXaa
115120125
GluProGlyIleGlySerSerLeuGluSerAsnAsnLysProLeuLeu
130135140
XaaSerXaaIleIleLeuValTyrLeuSerAsnIlePheSerIleGly
145150155160
IleGlyValIleSerMetSerProIleSerGlyAsnGlyValValVal
165170175
ValValIleSerSerLeuLeuValValValValValValGlyThrVal
180185190
ValValValValValAlaPheValValValValValThrIleGly
195200205
PheAsnValValValValAlaIleGlyGlyLeuProGlyThrPhe
210215220
ValValValValValGlyPheXaaValValValValMetIleGly
225230235240
LeuArgValValValValValAlaLeuAspSerGlySerSerSer
245250255
TrpPheAlaTrpThrSerSerCysSerSerCysTrpLeuSerCysGly
260265270

SerSerCysSerArgLysCysCysTyrGluIleSerXaaSerLeuIle
275280285
SerArgValAsnCysThrIleLeuArgSerThrSerValAlaIleAsn
290295300
PheIleGluXaaLeuArgArgLeuTyrTrpSerThrTyrXaaArgAsp
305310315320
IleLeuArgThrValTrpAlaTyrValAsnPheSerIleAlaAlaHis
325330335
ThrAsnTyrTyrXaaThrAlaValMetAlaSerTyrTrpArgAsnCys
340345350
GluIleGlnThrSerIleTrpGluLeu
355360
(2) INFORMATION FOR SEQ. . . NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are
identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
PheArgGlnLeuMetProAsnAsnGlnLeuArgLeuAlaArgGlyGly
51015
AsnLeuCysXaaGlnValGlnGluIleSerGlnGluSerArgMetLeu
202530
HisXaaThrGlnGlnProValProGlnValCysXaaThrVal
354045
(2) INFORMATION FOR SEQ. . . NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are
identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
IleProAlaIleAsnAlaLysGlnSerAlaGlnIleSerXaaArgTrp
51015
LysSerMetLeuThrSerProGlyAspLysProGlyValAlaAsnVal
202530
AlaLeuAsnSerAlaAlaSerSerThrSerValLeuAspSer
354045
(2) INFORMATION FOR SEQ. . . NO:11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are
identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:
AsnSerGlyAsnXaaCysGlnThrIleSerSerAspXaaLeuGluVal
51015
GluIleTyrValAsnLysSerArgArgXaaAlaArgSerArgGluCys

202530
CysIleLysLeuSerSerGlnPheHisLysCysValArgGlnTyr
354045
(2) INFORMATION FOR SEQ. . . NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:
IleLeuSerAsnThrLeuValGluLeuAlaAlaGluPheAsnAlaThr
151015
PheAlaThrProGlyLeuSerProGlyLeuValAsnIleAspPheHis
202530
LeuXaaLeuIleXaaAlaAspCysLeuAlaLeuIleAlaGlyIle
354045
(2) INFORMATION FOR SEQ. . . NO:13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:
ThrValXaaHisThrCysGlyThrGlyCysXaaValXaaCysAsnIle
151015
ArgAspSerTrpLeuIleSerTrpThrCysXaaHisArgPheProPro
202530
LeuAlaAsnLeuSerXaaLeuPheGlyIleAsnCysArgAsn
354045
(2) INFORMATION FOR SEQ. . . NO:14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:
TyrCysLeuThrHisLeuTrpAsnTrpLeuLeuSerLeuMetGlnHis
151015
SerArgLeuLeuAlaTyrLeuLeuAspLeuLeuThrXaaIleSerThr
202530
SerSerXaaSerGluLeuIleValTrpHisXaaLeuProGlu
354045
(2) INFORMATION FOR SEQ. . . NO:15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 350 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GlyAsnTyrProSerLeuLeuLeuArgAspIleThrGluAspIleXaa
51015
ValGlnAsnLysLeuThrLeuAsnGlnXaaIleGlnPheValAsnLeu
202530
IleXaaPheProValLysXaaAsnIlePheTyrLysIleArgTyrTyr
354045
SerIleLeuLysTyrThrIleValTyrXaaAsnGluTyrIleSerXaa
505560
LysValSerThrLysLeuTyrCysXaaSerCysTyrGlyArgTrpPhe
65707580
IleGlnGluGlyLeuLeuGluArgLeuGlnArgLysIleArgCysIle
859095
ValLeuLeuSerIleLysLeuHisIleCysMetSerIleXaaAsnLeu
100105110
SerIleGlnSerSerThrLysArgLeuXaaGluIleGlySerThrAla
115120125
ProLeuValCysSerValAspSerGlnTyrSerHisAlaAlaTrpArg
130135140
ArgThrProLeuGluGlnGlyGlyIleGlyProValAsnPheProLeu
145150155160
IleSerAspSerSerHisSerIleSerLysAsnTyrGlyValLeuSer
165170175
ArgGlyArgArgTyrCysSerGlnArgPheIleHisHisXaaGlnGly
180185190
GlySerArgCysSerPheXaaSerAsnLeuXaaLeuThrIleArgLys
195200205
IleSerArgArgAsnSerThrCysTyrXaaCysThrSerIleHisXaa
210215220
AsnLeuTrpXaaSerLeuProSerLysLeuGluGluGlyProLysArg
225230235240
AsnValSerTyrSerXaaArgCysPheGlnLeuSerXaaGlyLeuIle
245250255
LeuGluXaaPheAsnPheSerAsnGluProAsnPhePheLeuIleXaa
260265270
LeuPheLeuCysSerTyrLysSerAspAlaAsnGluTyrArgArgLeu
275280285
HisIleXaaIleLeuCysGlyAspXaaIleValGluXaaValGlnIle
290295300
AsnProGlyValValAsnValValLeuAsnPheCysAsnLeuSerPhe
305310315320
PhePheLeuLeuThrTyrLeuSerTrpCysXaaGlnSerSerIleArg
325330335
AsnHisTyrSerSerSerLysAlaGlyArgArgSerProXaa
340345350

(2) INFORMATION FOR SEQ. . . NO:16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 350 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ArgGluLeuProLeuIleIleAlaSerArgTyrAsnXaaGlyTyrLeu
51015

GlyThrLysXaaIleAspLeuGluSerIleAsnSerIleCysGluPhe
202530
AsnLeuIleSerSerLysValLysTyrPheLeuGlnAsnSerLeuLeu
354045
PheHisPheLysValTyrAsnSerLeuLeuLysXaaValHisXaaLeu
505560
GluSerXaaHisGlnThrLeuLeuLeuLysLeuLeuTrpGlnMetVal
65707580
HisSerArgArgSerProXaaAlaThrThrGluGluAsnThrLeuTyr
859095
CysSerSerIleHisXaaThrSerHisLeuTyrValHisLeuLysSer
100105110
XaaHisSerIleLysHisLysLysThrLeuArgAsnTrpGluTyrSer
115120125
SerSerArgValLeuSerXaaPheSerIleLeuProCysCysMetGlu
130135140
ThrTyrSerSerXaaThrArgTrpAsnTrpThrSerGlnPheProThr
145150155160
TyrLeuXaaLeuIleSerPheAsnXaaGlnGluLeuTrpCysThrPhe
165170175
SerArgLysLysValLeuLeuSerGluValTyrSerSerLeuThrArg
180185190
ArgValAlaLeuPheValLeuLysXaaSerMetThrTyrHisXaaGlu
195200205
AspGlnSerLysLysLeuTyrValLeuLeuMetHisPheAsnSerLeu
210215220
LysProMetValLysPheAlaGlnGlnThrGlyArgArgAlaLysLys
225230235240
GluCysGlnLeuLeuMetLysValPheProValIleLeuArgThrHis
245250255
PheArgMetIleXaaPhePheLysXaaThrLysPhePhePheAsnLeu
260265270
ThrPhePheMetXaaLeuXaaIleArgCysLysXaaValSerSerSer
275280285
ProHisLeuAspProLeuTrpArgLeuAspCysGlyIleGlyAlaAsn
290295300
LysProTrpSerCysXaaCysSerValLysPheLeuXaaPheIlePhe
305310315320
PhePheLeuIleAsnLeuProPheLeuValLeuAlaValPheTyrXaa
325330335
LysSerLeuLeuPhePheGlnGlyArgLysLysValSerLeu
340345350
(2) INFORMATION FOR SEQ. . . NO:17:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 351 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are
identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:
ProGlyIleThrProHisTyrCysPheGluIleXaaLeuArgIlePhe
51015
ArgTyrLysIleAsnXaaProXaaIleAsnLysPheAsnLeuXaaIle
202530
XaaPheAsnPheGlnXaaSerGluIlePhePheThrLysPheAlaIle
354045
IleProPheXaaSerIleGlnXaaSerIleGluMetSerThrLeuVal
505560
ArgLysLeuAlaProAsnPheThrAlaGluAlaValMetAlaAspGly
65707580
SerPheLysLysValSerLeuSerAspTyrArgGlyLysTyrValVal

859095
LeuPhePheTyrProLeuAsnPheThrPheValCysProSerGluIle
100105110
LeuAlaPheAsnGlnAlaGlnLysAspPheGluLysLeuGlyValGln
115120125
LeuLeuSerCysAlaGlnLeuIleLeuAsnThrProMetLeuHisGly
130135140
AspValLeuLeuLeuAsnLysValGluLeuAspGlnSerIleSerHis
145150155160
LeuSerLeuThrHisLeuIleGlnLeuAlaArgThrMetValTyrPhe
165170175
LeuGluGluGluGlyIleAlaLeuArgGlyLeuPheIleIleAspLys
180185190
GluGlyArgValValArgSerGluValIleTyrAspLeuProLeuGly
195200205
ArgSerValGluGluThrLeuArgValIleAspAlaLeuGlnPheThr
210215220
GluThrTyrGlyGluValCysProAlaAsnTrpLysLysGlyGlnLys
225230235240
GlyMetSerAlaThrHisGluGlyValSerSerTyrLeuLysAspSer
245250255
PheXaaAsnAspLeuIlePheGlnMetAsnGlnIlePhePheXaaSer
260265270
AspPhePheTyrValValIleAsnGlnMetGlnMetSerIleValVal
275280285
SerThrSerArgSerSerValAlaThrArgLeuTrpAsnArgCysLys
290295300
XaaThrLeuGluLeuLeuMetXaaCysXaaIlePheValIleTyrLeu
305310315320
PhePheSerTyrXaaLeuThrPheLeuGlyValSerSerLeuLeuLeu
325330335
GluIleIleThrLeuLeuProArgProGluGluGlyLeuLeuGlu
340345350
(2) INFORMATION FOR SEQ. . . NO:18:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 351 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:
LeuLysGluThrPhePheArgProTrpLysLysSerAsnAspPheXaa
151015
XaaLysThrAlaAsnThrLysLysGlyLysLeuIleArgLysLysLys
202530
IleAsnTyrLysAsnLeuThrLeuHisXaaGlnLeuGlnGlyLeuPhe
354045
AlaProIleProGlnSerSerArgHisArgGlySerArgCysGlyAsp
505560
AspAspThrHisLeuHisLeuIleTyrAsnTyrIleLysLysValArg
65707580
LeuLysLysAsnLeuValHisLeuLysAsnXaaIleIleLeuLysXaa
859095
ValLeuLysIleThrGlyAsnThrPheMetSerSerXaaHisSerPhe
100105110
LeuAlaLeuLeuProValCysTrpAlaAsnPheThrIleGlyPheSer
115120125
GluLeuLysCysIleAsnAsnThrXaaSerPhePheAspXaaSerSer
130135140
XaaTrpXaaValIleAspTyrPheArgThrAsnAsnAlaThrLeuLeu
145150155160

ValAsnAspGluXaaThrSerGluSerAsnThrPhePheLeuGluLys
165170175
ValHisHisSerSerCysXaaLeuAsnGluMetSerGlnArgXaaVal
180185190
GlyAsnXaaLeuValGlnPheHisLeuValGlnGluGluTyrValSer
195200205
MetGlnHisGlySerIleGluAsnGlnLeuSerThrArgGluGluLeu
210215220
TyrSerGlnPheLeuLysValPheLeuCysLeuIleGluCysXaaAsp
225230235240
PheArgTrpThrTyrLysCysGluValXaaTrpIleGluGluGlnTyr
245250255
AsnValPheSerSerValValAlaGlnGlyAspLeuLeuGluXaaThr
260265270
IleCysHisAsnSerPheSerSerLysValTrpCysXaaLeuSerAsn
275280285
XaaCysThrHisPheAsnArgLeuLeuTyrThrLeuLysTrpAsnAsn
290295300
SerGluPheCysLysLysTyrPheThrLeuLeuGluIleLysLeuAsn
305310315320
SerGlnIleGluPheIleAspSerArgSerIleTyrPheValProLys
325330335
TyrProGlnLeuTyrLeuGluAlaIleMetArgGlyAsnSerArg
340345350
(2) INFORMATION FOR SEQ. . . NO:19:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 350 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:
GlnGlyAspLeuLeuProAlaLeuGluGluGluXaaXaaPheLeuIle
151015
GluAspCysXaaHisGlnGluArgXaaValAsnLysLysLysLysAsp
202530
LysLeuGlnLysPheAsnThrThrLeuThrThrProGlyPheIleCys
354045
ThrTyrSerThrIleXaaSerProGlnArgIleXaaMetTrpArgArg
505560
ArgTyrSerPheAlaSerAspLeuXaaLeuHisLysLysSerGlnIle
65707580
LysLysLysPheGlySerPheGluLysLeuAsnHisSerLysMetSer
859095
ProXaaAspAsnTrpLysHisLeuHisGluXaaLeuThrPheLeuPhe
100105110
GlyProSerSerSerLeuLeuGlyLysLeuHisHisArgPheGlnXaa
115120125
IleGluValHisGlnXaaHisValGluPheLeuArgLeuIlePheLeu
130135140
MetValSerHisArgLeuLeuGlnAsnGluGlnArgAspProProCys
145150155160
GlnXaaXaaIleAsnLeuXaaGluGlnTyrLeuLeuProArgGluSer
165170175
ThrProXaaPheLeuLeuIleGluXaaAspGluSerGluIleSerGly
180185190
LysLeuThrGlyProIleProProCysSerArgGlyValArgLeuHis
195200205
AlaAlaTrpGluTyrXaaGluSerThrGluHisThrArgGlyAlaVal
210215220
LeuProIleSerGlnSerLeuPheValLeuAspXaaMetLeuArgPhe

225230235240
GlnMetAspIleGlnMetXaaSerLeuMetAspArgArgThrIleGln
245250255
ArgIlePheLeuCysSerArgSerArgArgProSerXaaMetAsnHis
260265270
LeuProXaaGlnLeuGlnGlnXaaSerLeuValLeuThrPheXaaLeu
275280285
MetTyrSerPheGlnXaaThrIleValTyrPheLysMetGluXaaXaa
290295300
ArgIleLeuXaaLysIlePheHisPheThrGlyAsnXaaIleLysPhe
305310315320
ThrAsnXaaIleTyrXaaPheLysValAsnLeuPheCysThrXaaIle
325330335
SerSerValIleSerArgSerAsnAsnGluGlyXaaPhePro
340345350
(2) INFORMATION FOR SEQ. . . NO:20:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 350 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:
SerArgArgProSerSerGlyLeuGlyArgArgValMetIleSerAsn
151015
ArgArgLeuLeuThrProArgLysValSerXaaXaaGluLysLysArg
202530
XaaIleThrLysIleXaaHisTyrIleAsnAsnSerArgValTyrLeu
354045
HisLeuPheHisAsnLeuValAlaThrGluAspLeuAspValGluThr
505560
ThrIleLeuIleCysIleXaaPheIleThrThrXaaLysLysSerAsp
65707580
XaaLysLysIleTrpPheIleXaaLysIleLysSerPheXaaAsnGlu
859095
SerLeuArgXaaLeuGluThrProSerXaaValAlaAspIleProPhe
100105110
TrpProPhePheGlnPheAlaGlyGlnThrSerProXaaValSerVal
115120125
AsnXaaSerAlaSerIleThrArgArgValSerSerThrAspLeuPro
130135140
AsnGlyLysSerXaaIleThrSerGluArgThrThrArgProSerLeu
145150155160
SerMetMetAsnLysProLeuArgAlaIleProSerSerSerArgLys
165170175
TyrThrIleValLeuAlaAsnXaaMetArgXaaValArgAspLysTrp
180185190
GluIleAspTrpSerAsnSerThrLeuPheLysArgSerThrSerPro
195200205
CysSerMetGlyValLeuArgIleAsnXaaAlaHisGluArgSerCys
210215220
ThrProAsnPheSerLysSerPheCysAlaXaaLeuAsnAlaLysIle
225230235240
SerAspGlyHisThrAsnValLysPheAsnGlyXaaLysAsnAsnThr
245250255
ThrTyrPheProLeuXaaSerLeuLysGluThrPheLeuAsnGluPro
260265270
SerAlaIleThrAlaSerAlaValLysPheGlyAlaAsnPheLeuThr
275280285
AsnValLeuIleSerIleAspTyrCysIleLeuXaaAsnGlyIleIle
290295300

AlaAsnPheValLysAsnIleSerLeuTyrTrpLysLeuAsnXaaIle
305310315320
HisLysLeuAsnLeuLeuIleGlnGlyGlnPheIleLeuTyrLeuAsn
325330335
IleLeuSerTyrIleSerLysGlnXaaXaaGlyValIlePro
340345350
(2) INFORMATION FOR SEQ. . . NO:21:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:
ThrLeuAsnGlnXaaIleGlnPheValAsnLeuIleXaaProVal
51015
LysXaaAsnIlePheTyrLysIleGlnTyrTyrSerIleLeuLysTyr
202530
ThrIleValTyrXaaAsnGluTyrIleSerXaaLysValSerThrLys
354045
LeuTyrCysXaaAlaValMetGlnMetValHisSerGluVal
505560
(2) INFORMATION FOR SEQ. . . NO:22:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:
AspLeuGluSerIleAsnSerIleCysGluPheAsnLeuIleSerSer
51015
LysValLysTyrPheLeuGlnAsnSerValLeuPheHisPheLysVal
202530
TyrAsnSerLeuLeuLysXaaValHisXaaLeuGluSerXaaHisGln
354045
ThrLeuLeuLeuSerCysTyrAlaAspGlySerPheArgGly
505560
(2) INFORMATION FOR SEQ. . . NO:23:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:
GlyProXaaIleAsnLysPheAsnLeuXaaIleXaaPheAsnPheGln
51015
XaaSerGluIlePhePheThrLysPheSerIleIleProPheXaaSer
202530
IleGlnXaaSerIleGluMetSerThrLeuValArgLysLeuAlaPro

354045
AsnPheThrAlaGluLeuLeuCysArgTrpPheIleGlnArgSer
505560
(2) INFORMATION FOR SEQ. . . NO:24:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:
ArgProLeuAsnGluProSerAlaXaaGlnLeuSerSerLysValTrp
151015
CysXaaLeuSerAsnXaaCysThrHisPheAsnArgLeuLeuTyrThr
202530
LeuLysTrpAsnAsnThrGluPheCysLeuLeuTyrPheThrLeuLeu
354045
GluIleLysLeuAsnSerGlnIleGluPheIleAspSerArgSer
505560
(2) INFORMATION FOR SEQ. . . NO:25:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:
ThrSerGluXaaThrIleCysIleThrAlaGlnGlnXaaSerLeuVal
151015
LeuThrPheXaaLeuMetTyrSerPheGlnXaaThrIleValTyrPhe
202530
LysMetGluXaaTyrXaaIleLeuXaaLysIlePheHisPheThrGly
354045
AsnXaaIleLysPheThrAsnXaaIleLysXaaPheLysVal
505560
(2) INFORMATION FOR SEQ. . . NO:26:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(ix) FEATURE:
(A) NAME/KEY: Positions coded by nonsense codons are identified as Xaa.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:
AspLeuXaaMetAsnHisLeuHisAsnSerSerAlaValLysPheGly
151015
AlaAsnPheLeuThrAsnValLeuIleSerIleAspTyrCysIleLeu
202530
XaaAsnGlyIleIleLeuAsnPheValLysAsnIleSerLeuTyrTrp
354045
LysLeuAsnXaaIleHisLysLeuAsnLeuLeuIleGlnGly
505560

(2) INFORMATION FOR SEQ. . . SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1086 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GCGAGTTCCCAGATAGAAGTTTGGATTTCACAATTCCTCCAGTAGCTGGCCATAACAGCT60
GTTCAATAATAGTTGGTGTGAGCGGCGATGGAAAAATTCACGTAAGCCCATACGGTTCTA120
AGGATGTCTCTCTAATAAGTGCTCCAATACAACCTTCTGAGTTATTCAATGAAGTTTATT180
GCGACACTTGTACTGCGAAGTATGGTGCAATTCACTCTGGATATCAAACTTCAGCTGATT240
TCGTAACAACACTTCCTACTACAACTGGAGCCGCAGGACAACCAACAACTACTACAACTG300
GAAGTCCAAGCAAACCAACTACTACTACCACTATCTAAGGCAACAACAACCACAACAACT360
CTTAATCCAATCATTACAACAACAACTCAAAAACCAACAACAACAACAACAACAAAGGTT420
CCAGGTAAGCCACCAATAGCCACAACAACAACAACATTAAAGCCAATAGTTACAACAACA480
ACAACAAAAGCAACAACAACAACAACAACAACAGTGCCAACGACAACTACTACTACCAAG540
AGAGACGAAATGACAACAACAACGACACCATTACCTGATATCGGTGACATTGAAATTACA600
CCAATCCCAATTGAAAAGATGTTGGATAAGTACACAAGAATGATTTATGACTATAACAGT660
GGTTTATTATTAGACTCTAATGATGAACCAATTCCAGGTTCTCAAGCAGGACAAATAGCT720
GATACAAGCAATTTATTCCCAGGTTCAAACTCACAAGAGTACTGGTTTACCAATTGATCC780
AATGGTTGGTCTTCCATTTGATCCAAAATCAGGTAATTTAGTACATCCATATACCAATCA840
AACAATGTCTGGTTTATCGGTATCATATCTTGCTGCTAAGAATTTGACAGTTGATACTGA900
TGAAACTACGGTTTACCAATTGATACACTCACTGGTTACCCATTGGATCCAGTCAGTTTG960
ATTCCGTTCAATCCAGAAACTGGTGAATTGTTTGTCCAATATCAGATGAGATAATGAATG1020
GAACAATTGCAGGTATTGTTTCAGGAATTTCTGCAAGTGAGTCATTATTATCTCAGAAAT1080
CGCTCC1086

(2) INFORMATION FOR SEQ ID NO:28:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1086 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum

CLM What is claimed is:

. . . 1. A hybrid vector comprising a regulatory DNA segment operably coupled to a DNA fragment encoding a polypeptide to which anti-**Cryptosporidium** antibodies specifically bind, wherein said sequence comprises SEQ ID NO 27 or SEQ ID NO 28.

. . . 2. The hybrid vector of claim 1, wherein said sequence further encodes a fusion protein comprising the polypeptide to which anti-**cryptosporidium** antibodies bind, operably coupled to another unrelated polypeptide sequence.

L4 ANSWER 13 OF 14 USPATFULL

ACCESSION NUMBER:	97:1169 USPATFULL
TITLE:	DNA sequence encoding surface protein of **cryptosporidium** parvum
INVENTOR(S):	Jenkins, Mark C., Bowie, MD, United States Fayer, Ronald, Ellicott City, MD, United States Tilley, Michael, Manhattan, KS, United States Upton, Steven L., Manhattan, KS, United States
PATENT ASSIGNEE(S):	The United States of America as represented by the Secretary of Agriculture, Washington, DC, United States (U.S. government) Kansas State University Research Foundation, Manhattan, KS, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 5591434		19970107

APPLICATION INFO.: US 1994-229393 19940415 (8)
RELATED APPLN. INFO.: Continuation-in-part of Ser. No. US 1993-68396, filed on 26 May 1993, now abandoned
DOCUMENT TYPE: Utility
FILE SEGMENT: Granted
PRIMARY EXAMINER: Caputa, Anthony C.
LEGAL REPRESENTATIVE: Silverstein, M. Howard, Deck, Randall E., Fado, John D.
NUMBER OF CLAIMS: 17
EXEMPLARY CLAIM: 12,14
NUMBER OF DRAWINGS: 4 Drawing Figure(s); 4 Drawing Page(s)
LINE COUNT: 900
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

TI DNA sequence encoding surface protein of **cryptosporidium** parvum

AB . . . for the immunization of animals against cryptosporidiosis. The Proteins are effective for the immunization of a variety of animals against **Cryptosporidium** parvum, particularly for the production of hyperimmune colostrum that may be used to confer passive immunity against the parasite. Isolated. . .

SUMM This invention relates to a novel protein for eliciting protective immune responses in animals against **Cryptosporidium** parvum, and recombinant DNA sequences which encode the protein.

SUMM The protozoan **Cryptosporidium** parvum, is spread by an oral-fecal route, infecting the intestinal epithelium and, to a lesser extent, the extraintestinal epithelia of. . .

SUMM . . . in calves, mice and humans, administration of hyperimmune bovine colostrum (HBC), prepared by immunizing cows with extracts of C. parvum **oocysts**, can effectively confer passive immunity against cryptosporidiosis [Fayer et al., J. Parasitol. 75(1):151-153 (1989); Fayer et al., J. Parasitol. 75(3):393-397. . .

SUMM . . . in the immunization of animals against cryptosporidiosis. The proteins are effective for the immunization of a variety of animals against **Cryptosporidium** parvum, particularly for the production of hyperimmune colostrum that may be used to confer passive immunity against the parasite. We. . .

DRWD FIG. 2. Northern and Southern blot hybridization of CP15/60 cDNA to formaldehyde gel-separated **Cryptosporidium** parvum total RNA and 0.8% agarose gel-separated C. parvum genomic DNA. RNA samples derived from three separate nucleic acid extractions. . .

DRWD FIG. 3. Immunostaining of Western blots containing native **Cryptosporidium** parvum sporozoite protein and purified recombinant CP15/60 protein. The primary immunoreagents were either monoclonal antibody 5C3, rat anti-native CP15 sera. . .

DETD . . . invention provides an isolated DNA sequence which encodes an antigenic protein effective for eliciting antibody production in an animal against **Cryptosporidium** parvum. The invention encompasses cDNA clones having a nucleotide sequence (SEQ ID No. 1) encoding rCP15/60 protein with the amino. . .

DETD Preparation of parasite nucleic acid and protein. **Cryptosporidium** parvum **oocysts** (2.times.10.sup.8 per preparation) were recovered from feces of cows during days 4-14 of a severe cryptosporidial infection. **Oocysts** were concentrated by sucrose flotation followed by purification over a cesium chloride gradient using the procedures described by Tilley et al. [Infect. Immun., 59(3):1002-1007, (1991)]. After centrifugation, **oocysts** were resuspended in 5.0 ml of 10 mM Tris-HCl pH 8.0, 1 mM EDTA (TE) and immersed drop-wise into a. . . are incorporated by reference herein]. DNA of C. parvum was prepared by treating the parasite extract with 1% sodium dodecylsulfate (**SDS**) and 50 ug/ml proteinase K (Gibco/BRL, Gaithersburg, Md.) in TE at 50.degree. C. for 2 h followed by phenol-chloroform extraction [Owen and Boorman, Nucl. Acid Res., 15(8):3631, (1987), the contents of which are incorporated by reference herein]. **Oocyst** protein was prepared by resuspending the parasite extract in 10 mM Tris-HCl pH 7.3, 1mM MgCl.sub.2 in the presence of. . .

DETD **Oocysts** of C. parvum were purified on CsCl gradients

[Taghi-Kilani and SeKal, Am. J. Trop. Med. Hyg., 36:505-508, (1987)] washed four times in PBS, freeze/thawed three times to release antigen. Proteins from 1.4.times.10.sup.9 **oocysts** were separated using 12% **SDS**-PAGE. A small vertical strip of the gel was excised and stained with Coomasie blue for visualization purposes. The strip was. . .

DETD . . . using random oligomers and Klenow DNA polymerase using manufacturer's instructions (Gibco/BRL). The cDNA probes were denatured by heating in a **boiling** water bath for 5 min, quick-chilled in an ice bath, and applied to both Southern and Northern blots that had. . . [Jenkins et al., Exp. Parasitol., 66:96-107, (1988)]. After 24 h hybridization with radiolabeled cDNA, blots were once with 2.times.SSC, 0.1% **SDS** at room temperature followed by three washes in 0.2.times.SSC, 0.1% **SDS** at 50.degree. C. After drying, blots were exposed to Kodak XAR film and stored at -70.degree. C. until development.

DETD . . . a 1.4 kb transcript (FIG. 2). The 1.4 kb RNA species was present in about equal concentrations from three separate **oocyst** extractions. Southern blot hybridization of CP15/60 to genomic DNA of C. parvum sporozoites showed single hybridizing bands in DNA digested. . .

DETD . . . sonication on ice for 20 sec, followed by DNase and RNase treatment. Expression of C. parvum cDNA was evaluated y **SDS** -PAGE followed by electrophoretic transfer to Immobilon paper (Millipore, Bedford, Mass.). Western blots containing recombinant and non-recombinant protein from uninduced and. . .

DETD Protein extracts from IPTG-induced E. coli containing either recombinant or non-recombinant plasmid were separated by preparative **SDS** -PAGE and transblotted to nitrocellulose membrane. Western blots were treated with PONCEAU S stain (Sigma) to visualize the recombinant protein which. . . non-recombinant protein was also excised. Both strips were cut into 0.5 cm pieces and placed in separate tubes containing 2.0% **SDS** and 1.0% TRITON-X 100 to elute the proteins from the paper. The eluted proteins were analyzed by **SDS**-PAGE followed by COOMASSIE BLUE staining or transblotting to nitrocellulose membrane and immunostaining with specific sera.

DETD Sporozoites were obtained by excystation of purified C. parvum **oocyst** using standard methods [Riggs and Perryman, Infect. Immun., 55(9):2081-2087, (1987)]. Excysted sporozoites were washed several times in PBS and then. . .

DETD . . . blots containing recombinant and control antigen with immune colostrum was performed to ensure that ELISA readings reflected anti-rCP15/60 responses. The **SDS**-PAGE and Western blotting procedure was identical to that described above.

DETD . . . 602 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: **Cryptosporidium** parvum
(D) DEVELOPMENTAL STAGE: Sporozoite
(vii) IMMEDIATE SOURCE:
(B) CLONE: CP15/60
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..474
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
GCACGAGTATTGATAAAAGAAAAACAAAACATGGGTAACTTGAAATCC48
AlaArgValLeuIleLysGluLysGlnAsnMetGlyAsnLeuLysSer
151015
TGTTGTTCTTTTGCCGATGAACACTCCCTAACCTCTACTCAACTAGTA96
CysCysSerPheAlaAspGluHisSerLeuThrSerThrGlnLeuVal
202530
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GTTGGAAATGGTTCAGGAGCTTCAGAAACTGCTTCCAACCACCCCCAA144
ValGlyAsnGlySerGlyAlaSerGluThrAlaSerAsnHisProGln
354045
GAAGAAGTTAATGATATCAATACTTTTAATGTAAAGTTAATAATGCAA192
GluGluValAsnAspIleAsnThrPheAsnValLysLeuIleMetGln
505560
GATAGAAGTAAGCTTGACTGCGAGGTAGTATTTGATAGCACAAGTATT240
AspArgSerLysLeuAspCysGluValValPheAspSerThrSerIle
65707580
TCGCTTTCTGGAGATGGAAAATGCAGAAATATTGCTTTGGATGAAATC288
SerLeuSerGlyAspGlyLysCysArgAsnIleAlaLeuAspGluIle
859095
CACCAATTATTATATTCAAAGGAAGAGCTTTCTAGAGTTGAAAGTAGT336
HisGlnLeuLeuTyrSerLysGluGluLeuSerArgValGluSerSer
100105110
GCTGGAATCAGCGATTCCGACAATTGTGTTGCAATTCATCTCAAAGAA384
AlaGlyIleSerAspSerAspAsnCysValAlaIleHisLeuLysGlu
115120125
TCAGGAAACTGTATTCCCCTTTTCTTTAATAATTCGCAAGACAAAGAA432
SerGlyAsnCysIleProLeuPhePheAsnAsnSerGlnAspLysGlu
130135140
AGATTTGTTGCAACAGCAAACAAATTCAAACCAAACTTTAAC474
ArgPheValAlaThrAlaAsnLysPheLysProAsnPheAsn
145150155
TAAAACAGGAATTTCATTAATTCTGGATTTGTTTTTTCCAAACCCTATAAATAAAATCCA534
AATAGCTTTTCTTCAATCACAGGGAGAGAATTAATAAAATATTTTTGATTAAAAAAAAAA594
AAAAAAAA602
(2) INFORMATION FOR. . .
CLM What is claimed is:
. . . through 158 of SEQ ID No. 2, wherein said protein is antigenic and effective to elicit an immune response against **Cryptosporidium** parvum in a host animal.

14. A method for protecting an animal against **Cryptosporidium** parvum comprising administering the isolated protein of claim 12 to said animal in an amount effective to elicit an immune response against **Cryptosporidium** parvum therein.

15. A method for producing hyperimmune colostrum containing antibodies to **Cryptosporidium** parvum comprising: (a) administering the isolated protein of claim 12 to an animal in an amount effective to elicit an immune response against **Cryptosporidium** parvum therein; (b) collecting hyperimmune colostrum containing antibodies specifics for **Cryptosporidium** parvum from said animal.

17. A method for protecting an animal against **Cryptosporidium** parvum comprising administering said hyperimmune colostrum of claim 15 to an animal.

L4 ANSWER 14 OF 14 USPATFULL

ACCESSION NUMBER:	96:27094 USPATFULL
TITLE:	Method of diagnosis of giardiasis using Giardia lamblia-specific stool antigen
INVENTOR(S):	Rosoff, John D., Mountain View, CA, United States Stibbs, Henry H., New Orleans, LA, United States
PATENT ASSIGNEE(S):	Alexon Biomedical, Inc., Mountain View, CA, United States (U.S. corporation)

	NUMBER	KIND	DATE
PATENT INFORMATION:	US 5503983		19960402
APPLICATION INFO.:	US 1993-66628		19930524 (8)
RELATED APPLN. INFO.:	Continuation of Ser. No. US 1989-330777, filed on 30 Mar 1989, now abandoned		
DOCUMENT TYPE:	Utility		

FILE SEGMENT: Granted
PRIMARY EXAMINER: Scheiner, Toni R.
ASSISTANT EXAMINER: Duffy, Patricia A.
LEGAL REPRESENTATIVE: Morrison & Foerster
NUMBER OF CLAIMS: 10
EXEMPLARY CLAIM: 1
NUMBER OF DRAWINGS: 6 Drawing Figure(s); 6 Drawing Page(s)
LINE COUNT: 896

DRWD FIG. 3 is a photograph of **SDS**-PAGE Western blots of affinity-purified GSA 65. Lane a contains protein molecular weight standards; lane b contains GSA 65 as detected. . .

DRWD FIG. 4 is a photograph of **SDS**-PAGE Western blots of proteolytically treated GSA 65. Lanes a and b, undigested (control) GSA 65; lane c contains trypsin-digested GSA. . .

DRWD FIG. 5 is a photograph of **SDS**-PAGE Western blots of heat-treated and oxidized GSA 65 as detected by 0.32% monospecific rabbit anti-GSA 65 antiserum. Lane a contains untreated (control) GSA 65; lane b contains GSA 65 **boiled** for 10 min; lane c, periodate-treated GSA 65, followed by NaBH.sub.4 reduction.

DETD Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS** -PAGE) was used for characterization of the G. lamblia-specific antigen in the immunoadsorbent column eluates and in G. lamblia trophozoite and cyst sonic extracts. **SDS**-PAGE was performed basically as described by Laemmli, U. K., Nature (London) 227:680-685 (1970). A 5% stacking gel and an 8%. . . of 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.2M Tris hydrochloride buffer (pH 6.8) and **boiled** for 2 min before addition to the sample slots. After electrophoresis, some unstained slabs were used for Western blotting. Others. . .

DETD Western blotting was performed by transferring antigens separated by **SDS**-PAGE to nitrocellulose paper. The antigens were analyzed by the method of Towbin et al., Proc. Natl. Acad. Sci. USA (1979). . .

DETD . . . exhaustively dialyzed against distilled water at 4.degree. C., and lyophilized or concentrated by ultrafiltration. Concentrates were analyzed by crossed immunoelectrophoresis, **SDS**-PAGE, and Western blotting.

DETD . . . acetone. Assay by immunofluorescence was also performed with smears of feces containing Blastocystis hominis or cysts of E. histolytica, with **oocysts** of Cryptospiridium purified from cattle, and with cysts of Chilomastix mesnili prepared from human stools by ethyl acetate-Formalin concentration as. . .

DETD . . . from cyst-positive stool eluates were individually passed over Sepharose affinity columns prepared with the IgG fraction of the monospecific antiserum. **SDS**-PAGE followed by Western blotting of reconcentrated affinity column eluates revealed that all of the eluates had identical banding patterns as. . .

DETD . . . and C. albicans. Fecal smears containing cysts of both E. histolytica and Entamoeba hartmanni and preparations of C. mesnili cysts, **Cryptosporidium oocysts**, and B. hominis were also nonreactive.

DETD Before characterization experiments, the purity of affinity-purified GSA 65 was assessed by **SDS**-PAGE, followed by Western blotting and silver staining. Nitro-cellulose sheets were developed with antitrophozoite antiserum and monospecific anti-GSA 65 antiserum to. . .

DETD The chemical nature of GSA 65 was characterized by proteolytic digestion, **boiling**, periodate oxidation, and lectin binding experiments. The antigen was **boiled** for 10 min to assess its heat stability. Stability under oxidizing conditions was assessed by periodate treatment: a solution of. . . followed by digestion at 60.degree. C. for 24 h. controls received 0.1M Tris hydrochloride alone. All digestions were terminated by **boiling** for 3 min. The effect of these treatments was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS**-PAGE), followed by Western blotting with monospecific rabbit anti-GSA 65 antiserum (infra).

DETD . . . retained the same Western blotting patterns as the undigested controls. GSA 65 was labile after periodate oxidation but stable after **boiling**. After oxidation with NaIO.sub.4 and reduction with NaBH.sub.4, GSA 65 lost immunoreactivity and could not be detected in immunoblots. After **boiling**, GSA 65 retained it immunoreactivity and electrophoretic mobility in Western blots. Controls showed no change in immunoreactivity, mobility, or molecular. . .

DETD GSA 65 resistance to proteolytic degradation and **boiling**. Its sensitivity to periodic acid oxidation suggest that this antigen contains carbohydrate. GSA 65 behaves as a glycoprotein in that. . . highly water soluble, precipitates in ammonium sulfate and trichloroacetic acid, and stains intensely with periodic acid-Schiff reagent in polyacrylamide gels. **SDS**-PAGE and IEF banding patterns confirmed that GSA 65 is a glycoprotein. Because of differential glycosidation, glycoproteins have a variable charge-to-mass. . .

DETD . . . Specimens with parasite | Specimens Tested in Each Category

Intestinal Parasite

Ascaris lumbricoides	9	1/9
Blastocystis hominis	3	0/3
Chilomastix mesnili	2	0/2
Clonorchis sinensis	1	0/1
Cryptosporidium	4	0/4
Entamoeba coli	9	1/9
Entamoeba hartmanni	2	0/2
Entamoeba histolytica	3	0/3
Endolimax nana	8	0/8
Hookworm	8	0/8
Hymenolepis nana	2	0/2
Iodamoeba buetschlii		

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